HISTOLOGIC BIOASSAY OF 3-METHYL-L-ASCORBIC ACID

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SIX FIGURES

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INTRODUCTION

In a previous report (Gould, Goldman and Clarke, '49) the findings of bioassays employing the serum phosphatase response and the histologic tooth structure examination indicated that 3-methyl-*l*-ascorbic acid is about one-fiftieth as active antiscorbutically as *l*-ascorbic acid and that 1-methyl-heteroascorbic acid showed strong antiscorbutic activity. It also confirmed the suggestion (Vestling and Rebstock, '46) that preliminary conversion of 3-methyl-*l*-ascorbic acid to *l*-ascorbic acid probably occurs, since both the blood phosphatase and histologic responses to the administration of large doses of 3-methyl-*l*-ascorbic acid were markedly delayed, in contrast to the prompt responses observed with *l*-ascorbic acid. It appeared that there was no biochemical antagonism between 3-methyl-*l*-ascorbic acid and *l*-ascorbic acid when mixtures of the two were fed, the former in relatively large and the latter in relatively small amounts.

The purpose of the present report is to present the histologic findings of the above-mentioned experiments and to demonstrate unequivocally the antiscorbutic properties of 3-methyl-*l*-ascorbic acid and its inability to compete with *l*-ascorbic acid metabolically.

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MATERIALS AND METHODS

Using the classical histologic bioassay technique to corroborate the phosphatase bioassay data on the question of whether 3-methyl-*l*-ascorbic acid shows any true antiscorbutic properties and whether it may act competitively with *l*-ascorbic acid, guinea pigs were kept on a scorbutogenic diet for 18 to 25 days and then fed various amounts of the compound. Other animals were kept on various doses of the compound for 18 to 25 days.

The midsagittal section of the head of the animal was cut in transverse sections at predetermined points at different levels of the incisor teeth. The decalcified (5% HNO_3) sections were cut, mounted and stained with hematoxylin and eosin. Some sections were also stained by Wilder's silver-stain method.

Method of interpretation of histopathologic findings

Höjer ('26) has proposed a bioassay method based on the observation that the degree of ascorbic acid deficiency can be estimated by histologic examination of the teeth. Using the cross section of the root showing the odontoblastic layer surrounding the pulp and the formation of dentin, Höjer described 10 stages in the development of scurvy, based on the amount of disorganization of the odontoblasts; the width, irregularity and structure of the dentin; and the degree of calcification of the predentin. A modification of this method was proposed by Boyle, Bessey, and Howe ('40), who based their assay on the width of the dentin layer of the incisor tooth; they found this varied with the vitamin C intake. Another assay described by Crampton ('47) was based on the length of the odontoblasts. He showed that in the normal guinea pig the odontoblasts at the formative end of the tooth increase in length until they reach maturity. At the senile end of the tooth, the odontoblasts have degenerated and are embedded in calcified tissue. Crampton found that in scorbutic animals the length of the odontoblasts varies with the intake

of ascorbic acid. With less than 0.5 mg ascorbic acid per day, the cells are so disorganized that accurate readings are difficult. In frank scurvy, Crampton felt that an assay could not be made.

In the present study the histopathologic technique to determine the state of scorbutus was based on the method used by Crampton and by Boyle et al. ('40), using one method to supplement the other. So that the determinations might be constant in order to compare one animal with another, the sections used for histologic examination were obtained by sectioning a hemi-section of the mandible just anterior and parallel to the first molar, at right angles to the longitudinal axis of the mandible. By this method comparison of sections of different animals could be made. Also confusion was eliminated as to the condition of the odontoblasts, since they degenerate normally at the incisal portion of the tooth, giving the same appearance as those which are present in the root in scorbutic conditions.

FINDINGS

Employing the conditions of the odontoblasts and the production of dentin as criteria of the scorbutic state of the animal, it was evident that 3-methyl-*l*-ascorbic acid fed at a level 25 times the critical dose of *l*-ascorbic acid necessary to maintain the phosphatase level showed no protective action, whereas when 50 times the critical dose was fed most of the animals were protected. To illustrate these findings, 4 typical animals were selected. Animal 24 was given a diet containing 0.5 mg/day *l*-ascorbic acid for 23 days. Examination for phosphatase showed 9.5 units (fig. 1). Previous investigation (Gould and Schwachman, '43) has established that a critical ascorbic acid intake — 0.225 mg/day — is necessary for normal osteoblastic function in the guinea pig, and the "alkaline" phosphatase level of the blood has been shown to be the most sensitive and earliest index of this function (Schwachman and Gould, '42; Gould and Schwachman, '42). Animal 14 was given a diet containing 5.6 mg/day 3-methyl-*l*-

ascorbic acid for 23 days; phosphatase fell to 2.6 units. Animal 15 was placed on the same diet as 14 but was then given 11.25 mg for 12 days; phosphatase fell to 4.2 units at 23 days and then rose after 12 days to 11.5 units. Animal 19 was given a diet containing 11.25 mg of the compound for 23 days; examination for phosphatase showed 9.5 units.

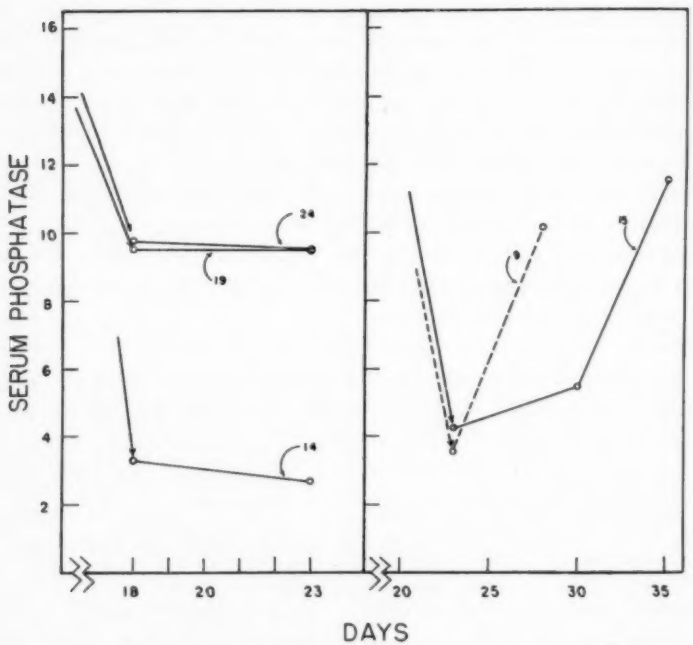
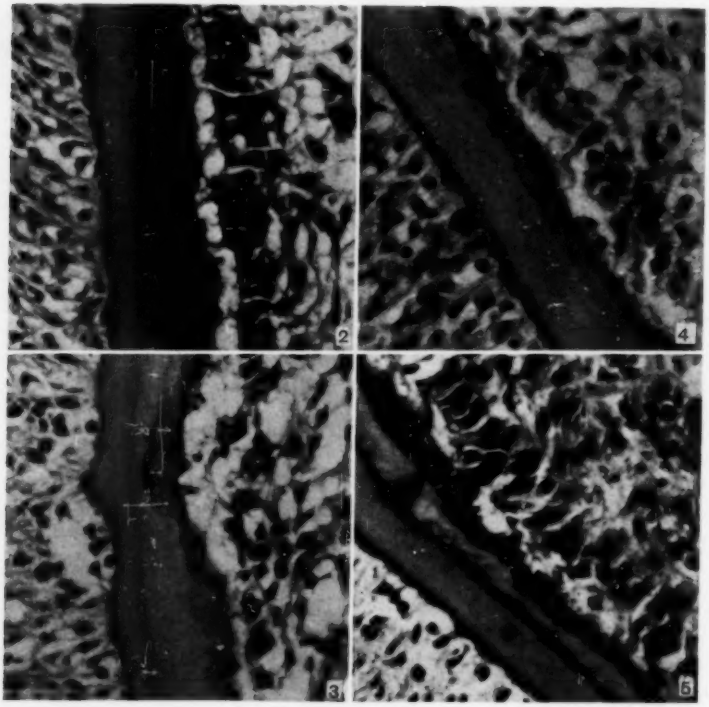


Fig. 1 Animals fed a scorbutogenic diet supplemented by: animal 24, 0.5 mg *l*-ascorbic acid; animal 19, 11.25 mg 3-methyl-*l*-ascorbic acid; and animal 14, 5.6 mg 3-methyl-*l*-ascorbic acid. Serum phosphatase is expressed in units each of which corresponds to the liberation of 1 mg phosphorus (as phosphate) by 100 ml of serum in one hour at 37°C., using glycerophosphate as substrate under the conditions of the experiment. Animal 9 was maintained on a scorbutogenic diet for 23 days, then fed 0.25 mg *l*-ascorbic acid for 5 days. Animal 15 was maintained on a scorbutogenic diet supplemented by 5.6 mg 3-methyl-*l*-ascorbic acid for 23 days, followed by an increase in the supplement to 11.25 mg daily for a further 12 days.

Thus a comparison can be made among the 4 animals; the first a protected animal, the second an animal given approximately *25* times the critical dose (0.225 mg) of *l*-ascorbic acid, the third an animal given approximately 25 times the critical dose of *l*-ascorbic acid for 23 days and then given 50 times the dose for 12 days, and the 4th animal given an amount of 3-methyl-*l*-ascorbic acid 50 times the critical dose of *l*-ascorbic acid.

Figures 2 to 5 show photomicrographs illustrating the findings based on observation of these 4 animals. The first photomicrograph (animal 24) illustrates the regularity of the odontoblastic layer, the height of the cells comparing favorably with that published by Crampton for an animal receiving 0.5 mg ascorbic acid daily. Dentin production is evident. The second photomicrograph (animal 14) shows cessation of dentin formation with no odontoblastic layer present. (This animal was evidently not protected by the 5.6 mg/day dosage of 3-methyl-*l*-ascorbic acid.) The third photomicrograph (animal 15) shows the presence of a somewhat regular odontoblastic layer, the cells being distinctly smaller than those in the 0.5 mg/day animal. A peripheral heavily stained layer of dentin is seen; an adjacent amorphous material is also seen, representing the starting up of dentin production. The trend of repair of the scorbutic condition can be noted. Comparison of the second and third photomicrographs is interesting in that, in the latter, reparative activity after 12 days of 11.25 mg/day of 3-methyl-*l*-ascorbic acid is evident. The 4th photomicrograph (animal 19) illustrates the status of the odontoblastic layer and dentin production in relation to the 0.5 mg/day animal — animal 24 — the first photomicrograph, and to animals given lesser dosages — animals 15 and 14 — second and third photomicrographs. In the 4th illustration the odontoblastic layer is more regular than in the second or third photographs but much less regular than in the first. Also the later dentin formation is of much poorer quality and smaller in amount than in the 0.5 mg/day animal but better than in the other two animals.

Thus animal 19, which received 11.25 mg daily throughout the feeding period, shows distinct changes indicative of the administration of an antiscorbutic substance but in amounts



Figs. 2–5 Photomicrographs showing the status of odontoblastic layer and dentin formation in 4 animals illustrating the effects of the following diets: (1) 0.5 mg/day *l*-ascorbic acid for 23 days; (2) 5.6 mg/day 3-methyl-*l*-ascorbic acid for 23 days; (3) 5.6 mg 3-methyl-*l*-ascorbic acid for 23 days followed by 11.25 mg/day for 12 days; and (4) 11.25 mg/day 3-methyl-*l*-ascorbic acid for 23 days.

definitely less than the equivalent of 0.5 mg/day ascorbic acid. The histologic bioassay methods do not permit an accurate estimate of levels less than 0.5 mg daily. It would appear, therefore, that these findings are not inconsistent with

those obtained using the phosphatase bioassay, where a dose of 11.25 mg of 3-methyl-*l*-ascorbic acid was found to be equivalent to 0.225 mg *l*-ascorbic acid.

To illustrate that there was no indication of an antagonistic relation between 3-methyl-*l*-ascorbic acid and *l*-ascorbic acid, guinea pig 22 was chosen. This animal was given a diet

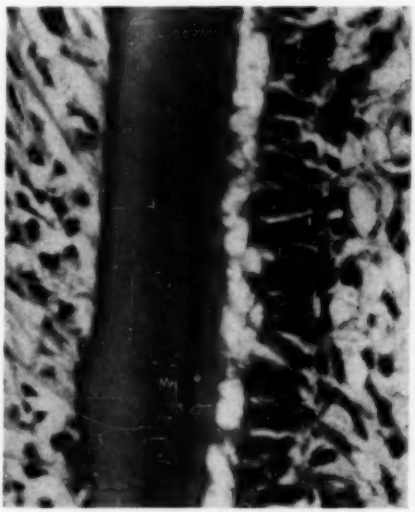


Fig. 6 Photomierograph showing the status of the odontoblastic layer and dentin production in an animal receiving a diet of 11.25 mg/day of 3-methyl-*l*-ascorbic acid and 0.5 mg/day of *l*-ascorbic acid. The appearance of the odontoblasts and dentin formation is similar to that of the 0.5 mg/day *l*-ascorbic acid animal seen in figure 2.

containing 11.25 mg/day of the former and 0.5 mg/day of the latter. Figure 6 shows the histologic findings. There is a regular odontoblastic layer and the height of the cells can be compared favorably with that seen in animal 24. Whether there is an additive effect brought about by the joint administration of the two substances could not be determined.

SUMMARY AND CONCLUSIONS

Histologic and enzymatic (phosphatase) data are presented to show that 3-methyl-*l*-ascorbic acid is unquestionably antiscorbutic but that the activity is, by histologic bioassay, definitely less than one-twenty-fifth of the activity of *l*-ascorbic acid and, on the basis of the phosphatase bioassay, in the order of about one-fiftieth of the activity of *l*-ascorbic acid.

There is no apparent competitive action between the metabolism of *l*-ascorbic acid and that of 3-methyl-*l*-ascorbic acid.

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DUODENAL ULCERS, LIVER DAMAGE, ANEMIA AND EDEMA OF CHRONIC CHOLINE DEFICIENCY IN DOGS

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SEVEN FIGURES

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The lipotropic action of choline on the livers of depancreatized dogs was discovered by Best, Ferguson, and Hershey ('33). Schaefer, McKibbin and Elvehjem ('41) demonstrated a requirement of choline for maximum early growth of young pups fed purified diets. Fouts ('43) observed fatty degeneration and cirrhosis of the liver and lowered hemoglobin in young pups and adult dogs receiving a diet high in fat and low in protein without supplementary choline. McKibbin, Thayer and Stare ('44) reported extensive studies of acute choline deficiency in pups fed various diets similar to those used in choline deficiency studies in rats by Engel and Salmon ('41). The production of anemia as well as of a nutritional edema in rats as a result of feeding diets low in protein and choline was described by Engel ('48).

For the past two years investigations have been conducted in this laboratory to determine whether or not chronic choline deficiency in dogs would produce neoplasms such as have been observed in rats (Copeland and Salmon, '46; Engel, Copeland and Salmon, '47; Staub, Viollier and Werthemann, '48; Schaefer, Copeland, Salmon and Hale, '50) and in chickens (Schaefer, Copeland and Salmon, '49). Since it was noted that vitamin B_{12} has a sparing action on the choline

requirement of the rat and chick (Schaefer, Salmon and Strength, '49a, b), it was of interest to determine if a similar effect could be demonstrated with the dog. In these experiments hematological and liver function changes were followed. This paper reports results in these studies, together with gross and histopathologic findings in dogs fed diets containing suboptimum amounts of choline for prolonged periods.

EXPERIMENTAL

Treatment of animals and diet

Three litters of weanling mongrel pups, 5 weeks of age, were used. Before being placed on experiment they were fed a stock diet with milk for 10 days; during this period they were dusted once with 10% DDT and dewormed twice with tetrachloroethylene. They were housed in metal metabolism cages equipped with large-mesh floors. The composition of the low-choline diets used is given in table 1. The extracted peanut meal and casein supplied a total of 0.30% methionine and 0.007% choline in the diet. Liver fraction "L," [1] when incorporated in the diet, supplied 0.005% choline. Feed and water were supplied ad libitum and the animals were weighed every third day.

Since it was desired to maintain dogs on a suboptimum choline intake for long periods, the dietary choline supplement was reduced at various intervals to determine how early and how drastically this reduction could be made. Some choline was initially included in the diets of all dogs, since McKibbin et al. ('44) had demonstrated that pups receiving a basal diet similar to diet 1d (table 1) failed to grow and died within three weeks. The amounts of choline initially supplied in the diets of all dogs were reduced at varying time intervals and in varying amounts, as is indicated in tables 2, 3, and 4, in which the summarized data are presented. One litter of pups was used in each of three experiments, which were started at 6-month intervals.

[1] Wilson's.

Methods

Blood samples were collected at approximately monthly intervals from the radial vein. Hemoglobin determinations were made according to the method of Evelyn ('36); hematocrit readings were obtained with Wintrobe tubes. The bromsulfalein test was slightly modified from that used by McKibbin et al. ('44). A blood sample was withdrawn prior to the dye

TABLE 1

Composition of diets

CONSTITUENTS	DIET			VITAMIN SUPPLEMENTS (mg/100 gm diet)	
	1d	2d	3d		
	%	%	%		
Peanut meal, extracted [1]	30.0	30.0	30.0	Thiamine	0.2
Sucrose	39.5	37.5	39.5	Pyridoxine	0.2
Casein, acid-washed [2]	6.0	6.0	6.0	Riboflavin	0.4
Salt mixture [2]	4.4	4.4	4.4	Calcium pantothenate	1.5
L-cystine	0.1	0.1	0.1	Niacin	3.0
Lard	19.0	19.0	19.0	Inositol	20.0
Cod liver oil	1.0	1.0	1.0	Folacin	0.1
Liver fraction "L" (Wilson)	..	2.0	..	Biotin	0.025
Vitamin B_{12} concentrate [3]	..	..	0.027	α-Tocopherol	2.5
				α-Tocopherol acetate	2.5
				2-methyl-1, 4-naphthoquinone	0.5

[1] Engel, R. W., J. Nutrition, *36:* 739, 1945.

[2] Salmon, W. D., J. Nutrition, *33:* 155, 1947.

[3] Vitamin B_{12} concentrate by Merck and Co., Rahway, N. J.; 0.027% supplied 15 μg per kilogram of diet.

injection and the plasma was used as a blank. Seven milligrams of bromsulfalein per kilogram of body weight were injected intravenously, and blood samples were obtained after intervals of 8 and 30 minutes. Determination of the dye in the plasma involved the addition of 2.5 ml of distilled water, 0.5 ml of plasma and 0.5 ml of normal NaOH to a cuvette of the Beckman spectrophotometer. The reading was made immediately at the absorption maximum of 580 mμ.

RESULTS

Experiment 1

It was found that supplementing the diet with 0.05% or less choline chloride resulted in death in 13 to 30 weeks. Even when the initial dietary choline supplement was 0.10% for 10 weeks, reduction of the dose resulted in rapid failure of the pup. It appeared that once the severe deficiency symptoms, such as drastic weight loss, anemia, and edema, developed, the feeding of choline chloride, DL-methionine, milk, or a sodium chloride-low diet did not appreciably prolong survival (table 2). Dogs 5 and 6, which were fed an initial choline chloride supplement of 0.30% for 27 weeks, served as controls for the early part of the experiment. Then the choline chloride was decreased to 0.05%. This reduction resulted in anemia, liver dysfunction, perforated ulcer, and the death of dog 6 after 25 weeks. Dog 5 maintained body weight fairly consistently from the 27th to 60th week; thereafter she gradually lost in body weight and developed impaired hepatic function.

Experiment 2

Since vitamin B_{12} had been shown to have a sparing action on the choline requirement of rats and chicks, diet 2d was fed to dogs 7 and 8 in experiment 2. This diet contained 2% of liver fraction "L," which supplied approximately 30 μg vitamin B_{12} per kilogram of diet. Hematological and hepatic dye clearance analyses were made at approximately monthly intervals. Pertinent data to indicate the effects of various treatments are presented in table 3.

As in experiment 1, the dogs receiving diet 1d plus 0.05% choline chloride developed liver dysfunction and cirrhosis, anemia and edema. The addition of a vitamin B_{12} concentrate or liver fraction "L" to the diet resulted in a marked protection against these symptoms.

Experiment 3

The effect of vitamin B_{12} was further investigated in experiment 3. Hematological and hepatic dye clearance data are given in table 4. The two dogs receiving vitamin B_{12} concentrate were normal at 54 weeks even though they received only 0.10% choline chloride supplement (dog 11) or 0.05% choline chloride after the 8th week (dog 12). Diet 1d supplemented with 0.60% choline chloride but without vitamin B_{12} supported normal hepatic function, blood formation, and growth for 54 weeks (dog 16). Dogs 13 and 14 fed diet 1d supplemented with 0.10% choline chloride grew at an initial rate comparable to that of dogs 11 and 12; hemoglobin was normal at 28 weeks, but hepatic dye clearance was slightly impaired. Reduction of the choline supplement at 34 and 28 weeks for dogs 13 and 14, respectively, further impaired hepatic dye clearance by the 54th week; however, hemoglobin remained normal. Dog 15, which was fed diet 1d plus 0.05% choline chloride, showed signs of impaired hepatic function at 20 weeks and died suddenly after 30 weeks on experiment.

Growth and survival

When dogs were fed diet 1d, the choline requirement for normal growth and maintenance of body weight was 0.10% or greater. Supplying 2% liver fraction "L" or a vitamin B_{12} concentrate in the diet reduced the choline requirement to 0.05%. One dog receiving diet 2d, which contained 2% liver fraction "L," appeared normal after receiving 0.05% choline chloride or less for 82 weeks. The response of dog 10 after receiving a vitamin B_{12} concentrate, which furnished 15 μg vitamin B_{12} per kilogram of diet, indicated that dogs require supplements of vitamin B_{12} with diets containing suboptimum amounts of choline.

In the absence of a source of vitamin B_{12}, 7 of 8 dogs died within 13 to 55 weeks after the choline supplement was reduced to 0.05%. This occurred whether the initial choline supplement had been 0.10% for 10 or 24 weeks or 0.3% for 27

TABLE 2

The influence of varying rates of choline feeding on body weight, hematology and liver function of dogs fed diet 1d in experiment 1

DOG No.	Sex	CHOLINE SUPPLEMENT % of diet	Time in weeks	BODY WT.	Hb	HEMATOCRIT	RBC	BROMSULFALEIN TEST 8 min.	BROMSULFALEIN TEST 30 min.	REMARKS
				lb.	*gm %*	*%*	*millions per mm³*	*μg dye/ml plasma*		
1	♀	0.05	16	21.5	14.9	36.3	6.6	..	..	Subnormal growth.
		0.025	2	17.7	6.6	19.6	3.1	..	..	Comatose condition. Fed orally 6 gm choline chloride and 300 ml milk.
		0.30	4	19.7	8.2	25.5	3.7	..	..	Resumed growth.
		0.05	7	26.2	3.4	13.0	2.6	78	44	Developed ascites.
		Died after a total of 30 weeks on experiment								Cirrhotic liver, ulcers.
2	♀	0.05	16	17.8	15.5	38.2	5.9	..	..	Subnormal growth.
		0.025	2	16.7	14.3	38.6	4.5	..	..	Decline in body weight.
		0.05	7	17.5	10.7	29.0	4.0	62	40	Ascites.
		0.05	4	22.2	8.1	22.0	3.8	82	45	Increase in ascitic fluid causing weight gain.
		0.05	2	22.1	3.2	10.0	2.3	..	..	Advanced ascites.
		Died after a total of 31 weeks on experiment								Cirrhotic liver and hyperplastic regeneration nodule, ulcers.
3	♀	0.10	10	21.6	14.7	39.2	6.6	..	..	Normal in appearance.
		0.00	8	18.5	11.0	38.7	4.6	..	..	Body weight loss.
		0.05	3	16.8	8.8	24.9	5.0	..	..	Further weight loss, no ascites.
		0.30	2	15.5	9.6	25.0	4.5	..	..	No response to treatment.
		Died after a total of 23 weeks on experiment								Perforated ulcer, diffuse fibrosis of the liver.
4	♀	0.10	10	15.0	15.2	40.0	6.2	..	..	Normal in appearance.
		0.00	8	17.2	9.6	26.0	3.6	..	..	Noticeable ascites.
										More advanced ascites.

		Died after a total of 23 weeks on experiment							No response to treatment. Perforated ulcer, diffuse fibrosis of the liver.
4	♀	0.10	10	15.0	15.2	40.0	6.2	..	..	Normal in appearance.
		0.00	8	17.2	9.6	26.0	3.6	..	..	Noticeable ascites.
		0.30	3	21.8	7.2	19.8	3.1	..	..	More advanced ascites.
		0.30	4	23.7	6.0	18.5	3.2	72	43	Attempts to correct ascites by adding 0.3% DL-methionine.
		0.30	2	23.1	6.2	19.0	4.4	65	42	Attempts to correct ascites by adding 0.3% DL-methionine and omitting NaCl from salt mixture. Ascites arrested.
		0.05	4	22.8	..	...	..	..	..	Attempts to correct ascites by adding 0.3% DL-methionine and omitting NaCl from salt mixture. Ascites arrested. Decline in weight.
		Died after a total of 31 weeks on experiment								Cirrhotic liver, ulcers.
5	♀	0.30	27	38.0	18.0	41.0	5.8	23	2	Normal.
		0.05	23	38.0	17.4	38.0	5.2	36	8	Normal.
		0.05	90	34.0	13.6	29.4	5.0	51	15	Gradual loss in body weight with advancing liver dysfunction.
6	♀	0.30	27	46.0	17.0	41.0	5.5	21	3	Normal.
		0.05	18	39.2	16.3	45.0	5.5	100 +	44	Rapid weight loss.
		0.05	4	34.0	8.4	23.0	3.6	100 +	48	Continued loss in weight.
		0.05	3	33.0	Died	...	..	..	..	Perforated ulcer, cirrhotic liver, ascites.

TABLE 3

The influence of varying rates of choline feeding on body weight, hematology and liver function of dogs in experiment 2

DOG No.	Sex	DIET	CHOLINE SUPPLEMENT % of diet	CHOLINE SUPPLEMENT Time in weeks	BODY WT.	Hb	HEMATOCRIT	RBC	BROMSULFALEIN TEST 8 min.	BROMSULFALEIN TEST 30 min.	REMARKS
					lb.	*gm %*	*%*	*millions per mm³*	*μg dye/ml plasma*		
7	♂	2d	0.10	10	37.0	14.0	32.1	5.1	17	3	Normal.
		2d	0.05	38	52.7	17.7	40.0	5.5	58	16	Normal appearance.
		2d	0.025	34	60.0	19.0	43.0	6.1	60	15	Normal appearance.
8	♂	2d	0.10	10	42.5	15.0	34.0	5.5	21	3	Normal.
		2d	0.05	20	51.0	15.5	36.0	5.0	13	3	Normal appearance.
		2d	0.025	18	55.5	15.5	38.3	5.4	35	9	Normal appearance.
		2d	0.00	34	60.0	17.5	42.0	6.6	38	10	Normal appearance.
9	♂	1d	0.10	24	48.6	13.2	30.0	4.3	18	2	Normal.
		1d	0.05	24	36.4	10.3	27.0	3.5	68	42	Sharp loss in body weight; edema.
		1d	0.05	4	39.4	6.5	18.0	3.0	80	46	Ascites, anorexia.
		1d	0.05	9	42.0	3.0	12.1	2.6	53	31	Injected 5 μg crystalline B_{12} daily for the first 10 days. Food consumption returned to normal. Slight improvement of liver function.
		1d	0.05	3	50.0	2.6	10.0	2.1	74	44	Jaundice, palpably enlarged liver.
		1d	0.05	15	57.0	2.5	10.0	2.3	100 +	56	Died. Ulcers, cirrhotic liver with hyperplastic regeneration nodule.
10	♂	1d	0.10	24	39.6	16.8	35.0	5.0	25	3	Subnormal growth.
		1d	0.05	12	32.3	15.4	32.0	5.3	48	20	Sharp loss in body weight, impaired hepatic function.
		3d	0.05	20	38.0	17.3	41.1	5.2	28	8	Weight gain and improved hepatic function.
		1d	0.05	4	40.0	17.7	40.0	6.8	27	4	Continued response.
		1d	0.05	20	39.0	18.7	51.0	6.3	44	15	Impaired hepatic function becoming evident.

TABLE 4

The influence of varying rates of choline feeding on body weight, hematology and liver function of dogs in experiment 3

DOG No.	DOG Sex	DIET	CHOLINE SUPPLEMENT % of diet	CHOLINE SUPPLEMENT Time in weeks	BODY WT.	Hb	HEMATOCRIT	RBC	BROMSULFALEIN TEST 8 min.	BROMSULFALEIN TEST 30 min.	REMARKS
					lb.	*gm %*	*%*	*millions per mm³*	*μg dye/ml plasma*		
11	♀	3d	0.10	8	7.8	11.1	30.0	4.5	7	3	Normal.
		3d	0.10	26	15.6	15.1	38.4	4.7	8	2	Normal.
		3d	0.10	20	17.0	15.3	39.0	5.6	14	6	Normal.
12	♂	3d	0.10	8	10.4	13.6	34.0	5.3	10	3	Normal.
		3d	0.05	26	17.6	17.2	43.1	5.9	18	4	Normal.
		3d	0.025	20	19.0	17.5	44.0	5.2	28	1	Normal.
13	♀	1d	0.10	8	7.3	11.5	30.0	5.1	12	2	Normal.
		1d	0.10	26	16.5	15.5	42.0	5.8	23	6	Normal.
		1d	0.05	20	18.0	15.9	40.0	6.3	90	27	Impaired liver function.
14	♂	1d	0.10	8	9.1	11.8	32.0	4.9	20	6	Normal.
		1d	0.10	20	20.1	14.3	33.0	5.9	27	9	Normal.
		1d	0.05	26	24.5	14.8	37.0	5.7	62	23	Impaired liver function.
15	♂	1d	0.05	8	10.6	12.8	33.0	5.4	10	2	Normal.
		1d	0.05	12	18.0	14.7	41.2	4.3	28	17	Normal.
		1d	0.05	8	19.4	13.3	34.0	5.5	67	32	Impaired liver function.
		1d	0.05	2	15.8	Died					Cirrhotic liver, hyperplastic regeneration nodule, and ascites.
16	♀	1d	0.60	8	9.3	10.7	23.0	4.2	4	0	Normal.
		1d	0.60	26	18.1	15.9	36.6	6.3	18	3	Normal.
		1d	0.60	20	19.5	15.6	42.0	5.9	20	2	Normal.

weeks. It was noted that in the advanced stages of the deficiency all the dogs were hyperirritable.

Liver function and anemia

Hepatic function became impaired in the chronically choline-deficient dogs 4 to 8 weeks before anemia developed. Terminal bromsulfalein retention in the choline-deficient dogs ranged from a dye concentration of 65 to 100 μg per milliliter of plasma at 8 minutes, and 32 to 48 μg at 30 minutes, as compared to the control values of 14 to 28 μg at 8 minutes and 1 to 6 μg at 30 minutes.

Of the dogs that died of chronic choline deficiency, three were severely anemic, having a hemoglobin concentration of 2.6 to 3.4 gm %, hematocrits of 10 to 13% and red blood cell counts of 2.1 to 2.6 million per mm^3 of blood. The other 4 dogs had lowered hemoglobin values ranging from 6.2 to 13.3 gm %, as compared to those of the control dogs of 15.1 to 17.7 gm %.

Edema and ascites

Marked subcutaneous edema and excessive fluid in the abdominal cavity were noted in 6 of the 7 animals that died of choline deficiency. Dog 4 contained as much as 4 l of serous fluid in the abdominal cavity. Four of the animals exhibited a severe ascites. One of these had a marked edema of the extremities, so that the legs were distended to 5 or 6 times their normal thickness. Although no specific attempt was made to correct the edema, various therapeutic procedures were tried in an effort to prolong life. Once ascites was evident, supplementation of diet 1d with 0.3% choline chloride and 0.3% DL-methionine did not arrest the further accumulation of abdominal fluid. This dietary supplementation in addition to the omission of sodium chloride from the diet seemed to arrest the condition. This therapy was not continued for prolonged periods. The inclusion of a source of vitamin B_{12} in the diet prevented the development of ascites in the cases studied.

Histological findings

Six of the 7 dogs had duodenal ulcers, and in the case of two dogs the ulcers were perforated. The largest ulcer measured 1.5 cm in diameter. The other 4 dogs had visible scars of healed ulcers and small ulcers visible microscopically, which involved the mucosa, muscularis mucosa, and submucosa.

A photomicrograph of a section from the edge of a perforated duodenal ulcer is shown in figure 1, and that of a section from a non-perforated ulcer in figure 2. The normal layers of the intestinal wall were replaced by zones of necrosis, fibrous tissue, and granulation tissue. The small ulcers involved the destruction of the mucosa and in some cases the muscularis mucosa and submucosa, but the muscularis externa and serosa were destroyed only in the cases of the two perforated duodenal ulcers. Excessive numbers of goblet cells were observed. Serial sections taken from different regions of the duodenum showed a distinctly abnormal mucosa. The ends of the secreting tubules were destroyed and replaced by accumulations of fibrous, inflammatory, and granulation tissue, as is illustrated in figure 3. In some cases the tubules had become cystic and contained what appeared to be a fibropurulent exudate (fig. 4). There was severe enteritis with hemorrhage in the duodenum, jejunum, and ileum in the case of dog 9.

Peritonitis was observed in the two dogs with perforated duodenal ulcers. Extensive acumulations of granulation tissue and endothelium-like cells were found scattered throughout the mesentery of each of these dogs.

The livers of all dogs appeared fatty upon gross examination. This was confirmed by determination of total liver lipid, which averaged 35% (dry basis), varying from 17.1 to 51.2%. The livers of 6 dogs were cirrhotic (figs. 5 and 6); 5 of these had extensive bile duct proliferation. The normal architecture of the liver was lost and pseudo-lobules were "walled off" with connective tissue. The interlobular tissue contained

large numbers of endothelium-like cells. Fat globules were scattered throughout the parenchyma. The nuclei were extremely basophilic and were frequently of odd sizes and shapes, with one or two distinct nucleoli. There were adenomatous lesions in the livers of three dogs which had some characteristics of hepatoma but were finally classified as hyperplastic regeneration nodules. The largest nodule measured 2 cm in diameter. The histology of this nodule is illustrated by figure 6. There was an abnormal arrangement of large parenchymal cells, with hyperchromatic nuclei of odd shapes. There was very little connective tissue stroma.

Microscopic studies of the kidneys revealed that some damage was present in all cases; however, true hemorrhagic degeneration was not observed. Some of the collecting tubules were dilated and portions appeared to contain vacuolated spaces (fig. 7). The glomeruli appeared congested with connective tissue and blood elements. There was some cloudy swelling and degeneration of the epithelial cells lining the convoluted tubules.

DISCUSSION

The choline requirement of dogs is greater than that for rats, as is indicated by the early failure of dogs fed diet 1d supplemented with 0.05% choline chloride. In the studies herein reported, 7 dogs died after receiving such diets for 13 to 55 weeks. Vitamin B_{12}, when supplied in the diets, prevented the deficiency symptoms that occurred in the dogs receiving suboptimum amounts of choline (0.05%).

McKibbin et al. ('44) observed that liver fraction "L" contains an unknown nutrient essential for dogs fed diets similar to 1d. Their diets were not supplemented with folacin and biotin; however, the inclusion of these nutrients in the diets used in the present experiments did not prevent failure. In view of the effectiveness of a vitamin B_{12} concentrate in replacing the liver fraction "L," and the results with chicks and rats (Schaefer et al., '49a, b) wherein crystalline vitamin B_{12} was the active component in sparing choline, the requirement of the dog for vitamin B_{12} when the diet contains

suboptimum amounts of choline is suggested. Since the deficiency symptoms in dogs receiving 0.05% supplementary choline chloride could be prevented, either by increasing the dietary choline or by supplying vitamin B_{12}, it appears that these nutrients are interrelated in the nutrition of the dog.

The appearance of an impaired hepatic function prior to the development of a severe anemia in choline-deficient dogs suggests that the anemia may be a result of this hepatic impairment.

Severe edema and ascites developed in dogs receiving a diet that furnished 20% + protein (15% supplied by the peanut meal and 5% + by casein). These symptoms could be prevented by supplementing the diet with 0.30% choline chloride or 0.05% choline chloride plus a vitamin B_{12} concentrate. This again emphasizes the vitamin B_{12}-sparing action on the choline requirement. Engel ('48) produced edema in rats on low-choline diets only when the diets were also low in protein. It is evident, however, that low-protein diets are not prerequisites for the production of edema in dogs as a result of low choline intake. Preliminary observations indicate that supplementing diet 1d with 0.30% choline chloride and 0.30% DL-methionine in addition to omitting the sodium chloride from the diet, was successful in arresting the edema.

The finding of duodenal ulcers in 6 of the 7 dogs that died of prolonged choline deficiency is of special interest. Whether these ulcers were specifically related to choline deficiency or were a secondary result of the accompanying inanition is not apparent.

Severity of liver damage was correlated with the total time the dogs survived on choline-deficient diets. This is illustrated by the mild liver damage characterized by fatty infiltration (51.2% fat, dry basis) and mild diffuse fibrosis in dog 3, which died after only 13 weeks on a low-choline diet, in contrast with the severe liver cirrhosis and extensive bile duct proliferation in dogs 1 and 6, which survived for 25 to 30 weeks on the low-choline diets. Three dogs that were fed

the low-choline diet for the longest period (30 to 55 weeks) had the most severe liver damage, characterized by hyperplastic regeneration nodules.

SUMMARY

1. Seven dogs died, after receiving a low-choline (0.05% choline chloride supplement) diet for 13 to 55 weeks. The livers of 6 of the dogs were cirrhotic and three of these livers had adenomatous lesions classified as hyperplastic regeneration nodules.

2. The choline-deficient animals had a severe hepatic impairment (bromsulfalein test) which preceded the development of anemia. In three of the deficient dogs hemoglobin, hematocrit and red blood cell counts were as low as 2.6 to 3.4 gm %, 10 to 13%, and 2.1 to 2.6 million mm^3 blood, respectively.

3. The prolonged feeding of diets low in choline but containing over 20% protein resulted in a severe nutritional edema and duodenal ulcers in 6 of 7 deficient dogs.

4. The requirement of the dog for vitamin B_{12} when the diet contains suboptimum amounts of choline was indicated. Vitamin B_{12}, when supplied in the diet, prevented the deficiency symptoms that occurred in the dogs fed diets supplemented with 0.05% choline chloride. When the diets were unsupplemented with vitamin B_{12}, the choline requirement for pups was 0.10% or greater.

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sulfalein by Hynson, Westcott and Dunning; and liver fraction "L" by the Wilson Laboratories.

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PLATE 1

EXPLANATION OF FIGURES

1 A photomicrograph of a section through the edge of a perforated duodenal ulcer. Hematoxylin and eosin. × 125.

2 A photomicrograph of a section through the edge of an ulcer that had not perforated. The normal layers of the intestinal well are replaced by zones of necrosis, fibrous and granulation tissue. Hematoxylin and eosin. × 125.

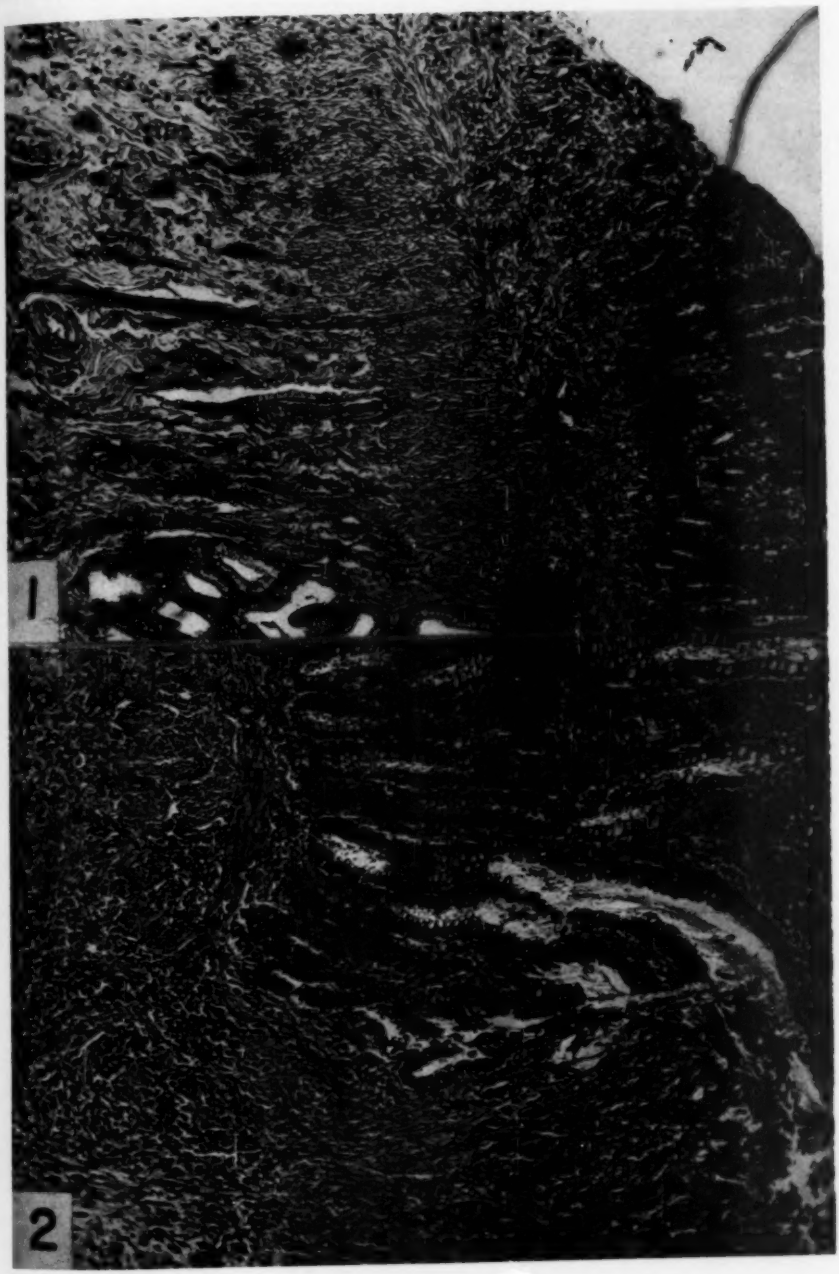
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PLATE 2

EXPLANATION OF FIGURES

3 Photomicrograph of a section of duodenum showing accumulation of fibrous, inflammatory and granulation tissue at the ends of the glandular tubules. There is an excessive number of goblet cells. Hematoxylin and eosin. × 125.

4 Photomicrograph of a section of duodenum showing a cystic condition of some of the glands. The cysts contain a fibro-purulent exudate. Hematoxylin and eosin. × 125.

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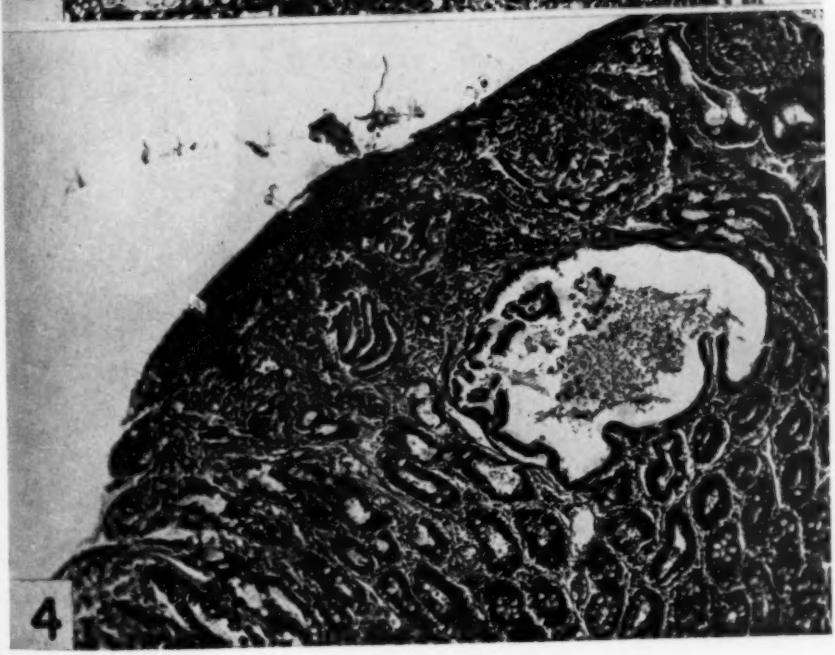
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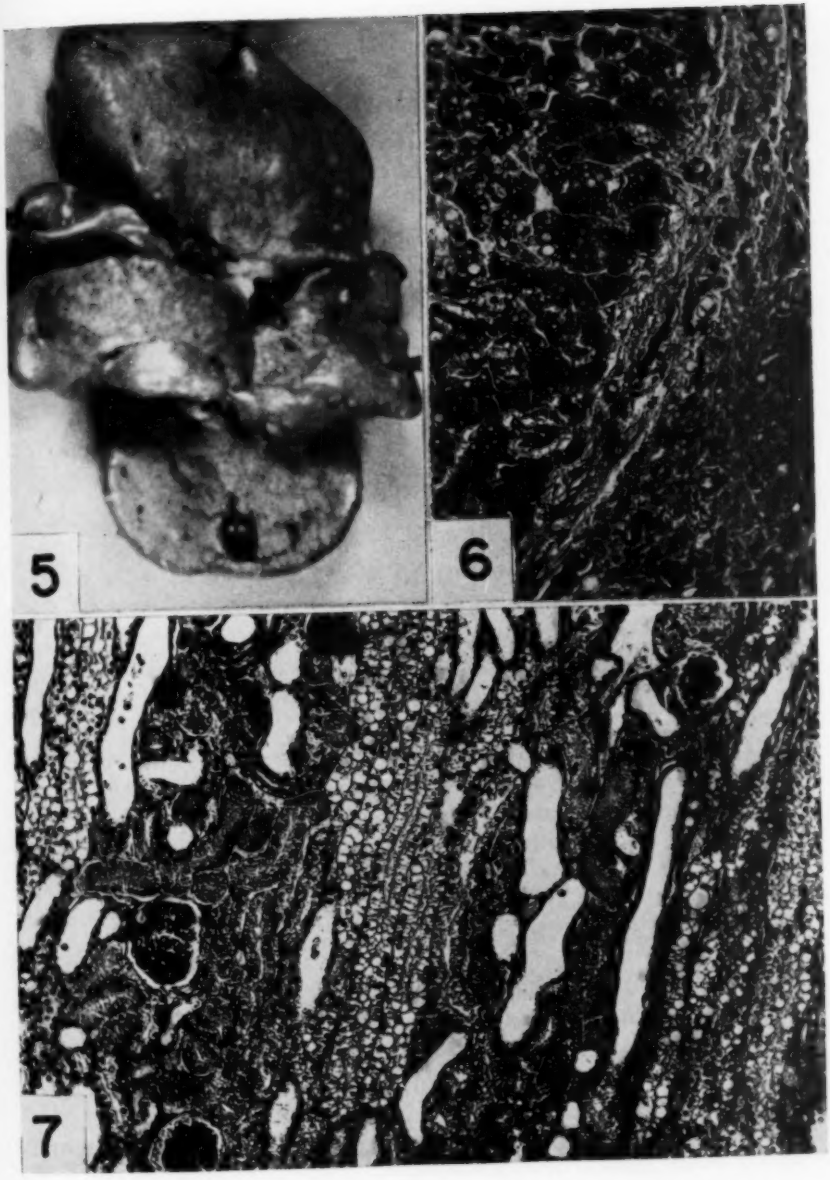
PLATE 3

EXPLANATION OF FIGURES

5 Photograph of cirrhotic liver from dog 9.

6 Photomicrograph of a section of the liver to illustrate the cellular nature of a part of one of the large regeneration nodules. Hematoxylin and eosin. × 210.

7 Photomicrograph of a section of kidney from a choline-deficient dog. Hematoxylin and eosin. × 125.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THE TRYPTOPHAN REQUIREMENT OF YOUNG TURKEY POULTS

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THREE FIGURES

(Received for publication September 18, 1950)

The need for tryptophan in the diet for the growth of chicks has been shown by Almquist and Mecchi ('41), Grau and Almquist ('44) and Wilkening et al. ('47), and the relationship of tryptophan to the nicotinic acid requirement was shown by Briggs ('45) and Briggs et al. ('46). However, there have been no reports on the tryptophan requirement of the turkey poult.

Several experiments were conducted in which turkey poults were fed rations containing various proteins which supply suboptimum levels of tryptophan. L- and DL-tryptophan were added to these rations at various levels to determine the relative activity of the D and L forms. The amount of DL-tryptophan required to give optimum growth was measured, from which it was possible to calculate the requirement of L-tryptophan.

EXPERIMENTAL

Methods

Bronze poults were fed a practical starting ration for one to two weeks before they were divided into experimental groups of equivalent weight. The poults were housed in electrically heated batteries with wire floors and were weighed at frequent intervals. The trials were continued for from 8 to 15 days. There were from 5 to 9 poults per group.

The basal ration [1] was similar to that described previously (Kratzer et al., '49) except that isolated soybean protein was replaced by other protein supplements as indicated. In all except 5 trials, 2% of condensed fish solubles was used to supply the animal protein factor. In experiments 3 and 4 in table 2 and in the experiments using meat scrap, gelatin and corn gluten feed, 0.25% of a commercial animal protein factor supplement [2] was added in place of fish solubles.

TABLE 1

Tryptophan and protein content of feed ingredients

MATERIAL	TRYPTOPHAN, CHEMICAL METHOD (Spices and Chambers, '49)	TRYPTOPHAN, MICROBIOLOGICAL METHOD	CRUDE PROTEIN (Kjeldahl)
	%	%	%
Casein	1.48		82.4
Oxidized casein	0.014	0.0128	82.0
Gelatin	0.014		92.9
Meat scrap	0.35	0.28	45.3
Corn gluten feed	0.174	0.1579	27.1
Condensed fish solubles	0.035		29.1

Oxidized casein was prepared by the method of Toennies ('42), modified to produce larger amounts of the treated casein in proportion to other reagents. The tryptophan content of the protein sources used was determined by the method of Spies and Chambers ('49). The microbiological method [3] was also used to determine the tryptophan content of some of the samples, for comparative purposes. The values obtained by these two methods are shown in table 1. Since the former

[1] Pteroylglutamic acid was kindly supplied by Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. T. H. Jukes. We are grateful to Merck and Co., Inc., Rahway, New Jersey, for other B complex vitamins supplied through the courtesy of Dr. D. F. Green.

[2] Lederle APF supplement was kindly furnished by Gillies and Loughlin, San Francisco, California.

[3] We are grateful to Mr. Ward Flach and Mr. C. D. Sander of the Eastern States Farmer's Exchange, Buffalo, New York, for making the microbiological assays using *lactobacillus arabinosus* after alkaline hydrolysis of the protein.

method does not require alkaline hydrolysis, in which some of the tryptophan is destroyed, it was felt that the values obtained by this method represented the tryptophan content of the feedstuffs most accurately. The DL- and L-tryptophan used were commercial preparations.

Poults which were fed rations deficient in tryptophan showed a reduction in growth rate within one day. At extremely low levels of tryptophan poults actually lost weight.

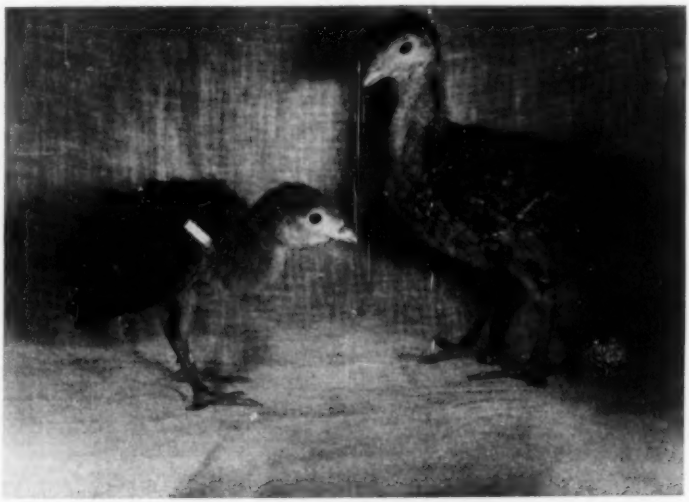

Fig. 1 Tryptophan-deficient and control poult after 9 days on experimental rations.

High mortality was noted in poults after approximately 10 days on a ration extremely low in tryptophan. No gross symptoms of the deficiency were noted with the exception of the failure to grow (see fig. 1).

Comparative values of D- *and* L-*tryptophan*

DL- and L-tryptophan were compared at various suboptimum levels in 4 different experiments. In experiments 1 and 2 the

basal ration contained 0.4% L-cystine, 0.5% DL-methionine, 12% gelatin, and 15% oxidized casein which supplied 24.9% crude protein. In these tests (see table 2) one group fed L-tryptophan was included which gave a rate of growth between that produced by two levels of DL-tryptophan. The amount of DL-tryptophan required to give a growth rate comparable to that obtained with a given level of L-tryptophan was deter-

TABLE 2

Relative effectiveness of L- *and* DL-*tryptophan in promoting growth in poults*

EXPERIMENT NO.	TYPE OF BASAL RATION	SUPPLEMENT	LEVEL	GAIN PER DAY	ACTIVITY, D-TRYPTOPHAN
			%	%	% *of* L-*Form*
1	Oxidized casein and gelatin	DL-tryptophan	0.2	0.88	
		DL-tryptophan	0.3	4.88	
		L-tryptophan	0.15	1.00	46
2	Oxidized casein and gelatin	DL-tryptophan	0.225	— 0.82	
		DL-tryptophan	0.3	4.70	
		L-tryptophan	0.15	0.83	21
3	Corn gluten feed, meat scrap and gelatin	None	...	1.55	
		DL-tryptophan	0.05	2.98	
		DL-tryptophan	0.1	5.70	
		L-tryptophan	0.035	3.87	
		L-tryptophan	0.07	5.68	23
4	Corn gluten feed, meat scrap and gelatin	None	...	1.63	
		DL-tryptophan	0.05	3.24	
		DL-tryptophan	0.1	4.88	
		L-tryptophan	0.03	2.98	
		L-tryptophan	0.06	4.75	27

mined by interpolation. The activity of D-tryptophan was calculated as per cent of L-tryptophan from the growth equivalents of the DL and L forms.

In experiments 3 and 4 the basal ration contained 0.05% DL-methionine, 0.1% L-lysine hydrochloride, 10.0% gelatin, 21.5% meat scrap and 30.0% corn gluten feed. The crude protein of this ration was 27.3%. In these experiments there were two groups fed each of the tryptophan isomers in addition to a

group fed the basal ration. Regression lines were calculated for the DL- and L-tryptophan response and equivalents were determined from these. The 4 experiments gave 46, 21, 23, and 27% activity, respectively, for D-tryptophan in relation to L-tryptophan. The approximate average of these values, or 30%, was used in converting DL activity to L activity in the experiments on the tryptophan requirement.

Oxidized casein and gelatin as protein sources

The amount of DL-tryptophan which was needed to supplement a basal ration containing 15.0% oxidized casein, 12.0% gelatin, 2.0% fish solubles, 0.5% DL-methionine, and 0.4% L-cystine was determined in experiments 5 and 6. The basal ration was calculated to contain 0.004% tryptophan and 24.9% crude protein. As is shown in figure 2, growth was increased from approximately — 3.5% gain per day to over 6% per day as the DL-tryptophan which was added was increased from zero to 0.4% of the ration. The L-tryptophan equivalent of the 0.4% DL-tryptophan which was needed for optimum growth was 0.26%, which would make the total tryptophan requirement in this experiment 0.264% of the ration.

Casein and gelatin as protein sources

Fifteen per cent crude casein, 12% gelatin, 0.4% cystine and 0.1% methionine were used in the basal ration in experiments 7 and 8. The basal ration contained 0.224% tryptophan and 24% crude protein. The results are shown in figure 2. In both experiments approximately 0.05% DL-tryptophan was required to produce optimum growth. Since the basal ration in this case contained a fairly high level of tryptophan, another experiment was conducted to determine if the small increases in growth with the additional tryptophan were significant. Duplicate groups of poults given the basal ration and 0.1% DL-tryptophan were fed for 12 days. The gain on the basal ration alone was 4.53% per day, while the growth of the groups supplemented with tryptophan was 6.87% per day. The dif-

ference was found to be significant at the 1% level, indicating that it was highly significant. The effect of the addition of 0.05% DL-tryptophan was not analyzed statistically. The L-tryptophan equivalent of the 0.05% DL-tryptophan which was required to supplement this basal ration was 0.033%, making a total requirement on this diet of 0.257% L-tryptophan.

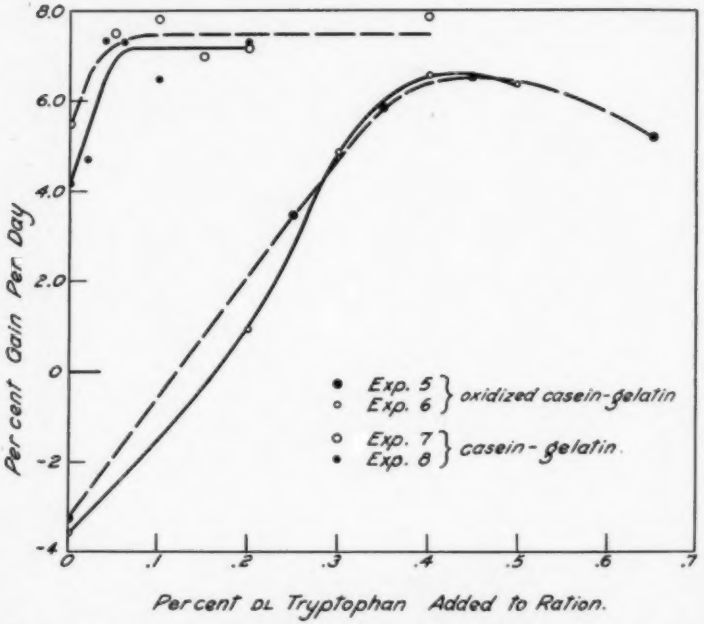

Fig. 2 Growth of poults fed various levels of DL-tryptophan added to rations containing gelatin and oxidized casein or untreated casein.

Corn gluten feed and gelatin as protein sources

In experiment 9 the ration contained 52.0% corn gluten feed, 10.0% gelatin, 2.0% fish solubles, 0.7% L-lysine hydrochloride, 0.1% L-arginine hydrochloride, 0.1% DL-methionine and 0.1% L-cystine. This ration contained 0.093% tryptophan and 25.0% crude protein. As is shown in figure 3, the growth increased

from approximately —0.5% to a 5% gain per day with an increase in DL-tryptophan from zero to 0.15%. The maximum rate of growth was slightly less than was obtained in experiments 5 to 8 and with practical rations. The L-tryptophan

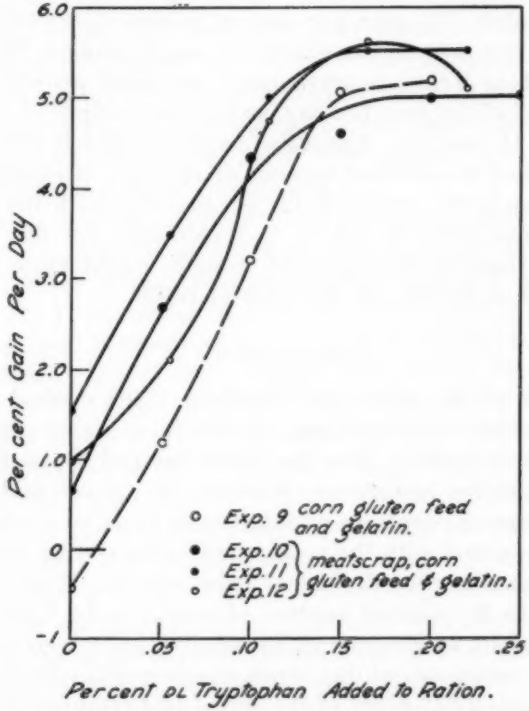

Fig. 3 Growth of poults fed various levels of DL-tryptophan added to rations containing corn gluten feed and gelatin, or meat scrap, corn gluten feed and gelatin.

equivalent of the 0.15% DL-tryptophan required to supplement the basal ration was approximately 0.098%, which would place the requirement with these protein sources at approximately 0.191% L-tryptophan.

Meat scrap, gelatin and corn gluten feed as protein sources

With 30% corn gluten feed, 10% gelatin, 21.5% meat scrap, 0.07% L-lysine hydrochloride and 0.05% DL-methionine in the ration, a tryptophan-low diet was prepared which produced approximately 1% gain per day in poults. The ration contained 0.129% tryptophan and 27.3% crude protein. The addition of about 0.15% DL-tryptophan increased growth to approximately 5.5% gain per day, as is shown in figure 3 (experiments 10, 11, and 12). Again the maximum growth was not as great as may be obtained on some other types of rations. The tryptophan in the ration (0.129%), together with the L-tryptophan equivalent of 0.15% DL-tryptophan, or the 0.098% which is required for optimum growth, would place the requirement at 0.227% on this type of ration.

DISCUSSION

The use of the Spies and Chambers ('49) method for the determination of tryptophan results in a higher value for crude casein (1.48%) than has been obtained by methods involving alkaline hydrolysis (Kratzer, '44; Green and Green, '43). The poult requirement determined by using crude casein agrees very well with the requirement obtained by the use of oxidized casein when the higher value is used. If one were to assume the tryptophan content of casein to be 1.2% of the crude protein, as determined by alkaline hydrolysis methods, the basal ration containing crude casein would contain 0.15% tryptophan. The 0.033% of the amino acid required to supplement the basal ration would give a requirement of 0.183% tryptophan. This is much lower than the requirement determined by using a basal ration containing oxidized casein but is only slightly lower than the requirement found when corn gluten feed and gelatin were the protein sources.

It is interesting to estimate the tryptophan content of the crude casein from the results of the feeding experiments, assuming that the amount in the oxidized casein is 0.014% as de-

termined chemically. The amount of DL-tryptophan required to supplement oxidized casein was 0.4% while only 0.05% was required to supplement crude casein. The difference of 0.35% between these two amounts of DL-tryptophan is equivalent to 0.2275% L-tryptophan, which represents the amount of tryptophan destroyed by the oxidative treatment. Since the basal rations contained 15% casein, we may calculate that the casein contained ($\frac{0.2275}{15} \times 100 + 0.014$), or 1.53% tryptophan. This is in close agreement with the analytical value of 1.48% and supports the belief that the method of Spies and Chambers gives a more accurate estimation of tryptophan than methods requiring hydrolysis of the protein.

The tryptophan requirements with rations containing crude casein or oxidized casein were found to be approximately 0.26% of the ration. When corn gluten feed and gelatin were used, the requirement was approximately 0.19%. With corn gluten feed, gelatin and meat scrap in the basal ration approximately 0.23% was needed. The reason for the differences in the requirement attendant upon using the various proteins is not known. It is doubtful that the availability of the tryptophan in the protein is an important factor. Schweigert ('48) found with chicks that the tryptophan in casein was completely available, while it was only partially available in fish meal, soybean oil meal and raw soybean meal. One would have to assume the tryptophan only partially available in casein and completely available in corn gluten feed and meat scrap if the present results were to be explanied on an availability basis. The slightly greater growth rate in the experiments in which casein was fed may have caused a slightly greater requirement for the amino acid. However, this was not observed to be true by Almquist ('47) in work with chicks.

The finding of 30% activity of D-tryptophan agrees with the work of Wilkening and Schweigert ('47) with chicks, in which they found activity varying from 17 to 40%. This is not in agreement with the earlier findings of Grau and Almquist ('44), which showed no activity for D-tryptophan.

The crude protein of the basal rations varied from 24.0 to 27.3% in the various trials. These levels are similar to those used in practical rations for starting poults. Within the small range in protein level there was no indication of a change in tryptophan requirement with protein level.[4]

No attempt was made to study the tryptophan-nicotinic acid relationship in the poult. It was felt that the nicotinic acid supplied in the ration was sufficient to meet the need for the vitamin, so that the tryptophan would not be used for the synthesis of nicotinic acid. The basal ration supplied 10 mg of nicotinic acid per 100 gm, which is greater than the minimum requirements found by Briggs ('46), Jukes et al. ('47), and Lance and Hogan ('48).

Assuming that the tryptophan requirement for young poults for rapid growth is 0.26% of the ration, the poult need is about the same as the requirement of 0.25% reported for the chick by Grau and Almquist ('44), but is greater than the 0.18% reported by Wilkening et al. ('47). One might expect a slightly greater need on the part of the poult, since it has a slightly greater growth rate and protein requirement than the chick.

SUMMARY

The tryptophan requirement of young turkey poults for growth was found to be approximately 0.26% of the ration when the basal ration contained gelatin and casein or oxidized casein. When corn gluten feed, meat scrap and gelatin, or corn gluten feed and gelatin were used, the requirement was approximately 0.23% and 0.19%, respectively. D-tryptophan was approximately 30% as active as L-tryptophan in promoting growth in poults.

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THE VITAMIN A REQUIREMENT OF THE TURKEY POULT FED A PURIFIED DIET[1,2,3]

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ONE FIGURE

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The vitamin A requirement of growing turkey poults fed a ration consisting of natural feedstuffs has been reported to be twice the chick requirement on the basis of the quantity of yellow corn needed in the ration (Scott and Hughes, '32; Scott, '37). Using crystalline carotene as a source of vitamin A, Wilgus ('40) found that poults require about 600 I.U. of the vitamin per 100 gm of diet for maximum growth and minimum mortality. After the present work was completed, Wharton, Matterson, Scott and Bliss ('49) reported the requirement to be 1,700 I.U. of the vitamin per 100 gm of feed.

Since numerous experiments have indicated that the absorption and storage of vitamin A may vary markedly with changes in the source of vitamin A and with the basal diet used (Gray, Hickman and Brown, '40; Gridgeman, Lees and Wilkinson, '40; Russell, Taylor, Walker and Polskin, '42), the

[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Agricultural Biochemistry.

[2] This study was made possible by a grant from The Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

[3] Presented before the American Institute of Nutrition, Detroit, Mich., April 18–22, 1949.

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present experiment was performed to determine the vitamin A requirement of growing poults fed a purified diet.

EXPERIMENTAL

Day-old poults of the Jersey Buff strain, housed in electrically heated brooders, were fed the purified diet described below for 9 days in order to deplete partially their stores of vitamin A.

The purified diet contained the following ingredients, expressed in per cent: casein (alcohol-extracted) 36.7; gelatin, 10.5; starch, 27.2; sucrose, 10.0; corn oil, 4.0; non-nutritive cellulose, 2.0; salt mixture,[5] 5.0; $CaHPO_4 \cdot 2H_2O$, 2.0; $CaCO_3$, 2.0; choline chloride, 0.2; inositol, 0.1 and para-aminobenzoic acid, 0.1.

The following vitamins were provided, in milligrams per 100 gm of diet: thiamine HCl, 1.0; pyridoxine HCl, 1.0; riboflavin, 2.0; calcium pantothenate, 2.0; niacinamide, 5.0; menadione, 2.5; mixed tocopherols (50% alpha, 15% beta, 22% gamma, 13% delta), 2.5; biotin, 0.04; folic acid, 0.2. Activated animal sterol supplied 280 A.O.A.C. units of vitamin D per 100 gm of diet.

After the depletion period the poults were distributed in 8 groups of 10 or 11 birds each, so that the average group weights were approximately the same. Six of the groups were then fed the purified diet supplemented with graded levels of vitamin A, provided as a distilled vitamin A concentrate in the natural ester form. One group served as the negative control (table 1) and the remaining group, which was utilized as a positive control, was fed a commercial turkey starting mash.

[5] The salt mixture, devised by A. G. Hogan (personal communication), was modified by decreasing the quantity of manganese sulfate and by substituting ferric ammonium citrate for $FePO_4 \cdot 4H_2O$. The quantities are expressed in grams: $CaCO_3$, 267; $CaHPO_4 \cdot 2H_2O$, 416; $MgCO_3$, 25; $MgSO_4 \cdot 7H_2O$, 30; NaCl, 127; KCl, 21; ferric ammonium citrate, 29.6; KH_2PO_4, 315; $MnSO_4 \cdot 4H_2O$, 5; $CuSO_4 \cdot 5H_2O$, 1.6; $Al_2(SO_4)_3$ $K_2SO_4 \cdot 24H_2O$, 0.24; KI, 0.6; $CoCl_2 \cdot 6H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.5; NaF, 1.0.

As the birds died they were examined to determine the cause of death, special attention being paid to the usual signs of vitamin A deficiency in fowl; namely, an opaque appearance of the third eyelid, lesions of the mucous membrane of the esophagus and crop, and an accumulation of urates in the ureters and in the kidney tubules, causing this organ to enlarge and become creamy white. Vitamin A deficiency was also judged on the basis of weight gains and external signs, including emaciation, staggering gait, and ruffled plumage.

TABLE 1

Growth response of turkey poults fed graded levels of vitamin A

GROUP [1]	RATION	VITAMIN A LEVEL	INITIAL WT., 10 DAYS OF AGE	WT., 4 WEEKS ON EXPERIMENT, 38 DAYS OF AGE			WT., 6 WEEKS ON EXPERIMENT, 52 DAYS OF AGE		
		I.U./100 gm	*gm*	*gm*	*S.E.*[4]		*gm*	*S.E.*[4]	
1	Purified	100	126 (11)[3]	495	± 42	(7)	821		(1)
2	Purified	200	129 (11)	600	± 34	(9)	849	± 71	(9)
3	Purified	300	126 (11)	675	± 42	(11)	1110	± 64	(10)
4	Purified	400	129 (11)	680	± 30	(11)	980	± 72	(10)
5	Purified	500	126 (11)	731	± 33	(10)	1189	± 66	(10)
6	Purified	600	126 (11)	714	± 33	(10)	1143	± 70	(10)
7	Starting mash	800 [2]	126 (11)	600	± 25	(8)	1065	± 46	(8)
8	Purified	None	122 (10)	All dead					

[1] The group numbers 1–6 indicate the level of vitamin A fed.

[2] Calculated value.

[3] Values in parentheses give number of birds in the group.

[4] S.E. (standard error) $= \frac{\text{S.D.}}{\sqrt{n}}$.

During the post mortem examination the livers were removed and stored frozen until vitamin A determinations could be made. After 6 weeks on experiment, several birds from each remaining group were sacrificed and the vitamin A contents of the livers determined by the Carr-Price reaction. A modified antimony trichloride reagent containing 2% acetyl chloride was employed. Nield, Russell and Zimmerli ('40) found this reagent to be effective in the determination of vitamins D_2 and D_3. It has also been shown that the addition of acetyl

chloride does not affect the intensity of the blue color developed in the reaction with vitamin A (A. Zimmerli, personal communication). The modified reagent has the advantage of nullifying the effect of small amounts of moisture which may be present.

RESULTS AND DISCUSSION

Growth

After 4 weeks on experiment (38 days of age) the poults of groups 3 (300 I.U.) and 4 (400 I.U.) showed the same weight gain, which was significantly higher than the gains at the lower levels of the vitamin. For higher levels of the factor, groups 5 (500 I.U.) and 6 (600 I.U.), the growth responses were somewhat greater than those for groups 3 and 4, but not significantly so.

After 6 weeks on experiment (52 days of age) the same weight gain relationships prevailed as after 4 weeks, except that the response of group 4 was significantly less than that at the next lower and next higher levels. In this group the range of individual weights was large, although none of the responses deviated sufficiently from the mean weight to eliminate them on a statistical basis. Further evidence that the growth response of group 4 was out of line was obtained in another experiment (unpublished data) in which poults fed 400 I.U. per 100 gm of a similar purified diet showed a growth response essentially the same as that obtained when 500 or 600 I.U. were supplied.

Although after 4 and 6 weeks on experiment the growth responses for groups 3 to 6, inclusive, were not statistically different, the responses for groups 5 and 6 were somewhat greater than those of the next lower groups. Therefore the minimum level of vitamin A which would provide for the growth needs of all members of a group would lie between 400 and 500 I.U. of the vitamin per 100 gm of diet, and a level of 450 I.U. is suggested by the above considerations.

The growth of groups 3, 5 and 6, it should be noted, was somewhat above that of group 7, which received a commercial

starting mash. Furthermore, the growth of the latter group was above that of poults of this strain raised under farm conditions, and it is apparent that the basal diet was adequate with respect to essential nutrients except for vitamin A.

To obtain a fair estimate of the vitamin requirements for growth, Hegsted ('48) has suggested that the log dose-response curve should be drawn through the region of approximately linear response. The vitamin requirement is then estimated from the point at which the regression line reaches the maximum growth response. Accordingly, the regression line was calculated from the weight gains for groups 1, 2, and 3 during the third and 4th weeks of the experiment, and the mean of the responses of groups 5 and 6 was taken as the maximum daily gain. This value amounted to 25 gm per day. The regression line (fig. 1) crosses the maximum response line at a point corresponding to a vitamin A level of approximately 450 I.U. per 100 gm of diet and therefore this method of treatment confirms the estimate of the minimum requirement given above.

Vitamin A deficiency signs and mortality

The first sign of vitamin A deficiency to be observed was concerned with the condition of the eyes. After 10 days on experiment (19 days on the vitamin A-deficient diet), several birds in group 8 (no vitamin A) developed a condition in which the inner eyelid became cloudy and remained partly closed. Occasionally a cheesy exudate around the eyes was noted. After 14 days on experiment, this eye condition was also observed in group 1 (100 I.U. vitamin A per 100 gm of diet).

All of the birds fed a diet completely devoid of vitamin A or containing 100 I.U. per 100 gm developed dry and ruffled plumage by the end of the second week of the experiment. Unsteadiness and a tendency to fall backward were also characteristics of these groups. The same deficiency signs became apparent in group 2 (200 units) after 4 weeks on experiment.

Several birds in groups 3 (300 units) and 4 (400 units) developed either slightly dry and ruffled plumage or slightly opaque inner eyelids, but none showed the severe eye conditions and unsteadiness characteristic of groups 1, 2, and 8.

One hundred per cent mortality occurred in group 8 within 16 days (after 25 days on the vitamin A-free diet), while 10 of the 11 birds in group 1 succumbed within 40 days on experiment. Only one death in group 2 and possibly one in group 3 could be attributed to a vitamin A deficiency. Several birds in

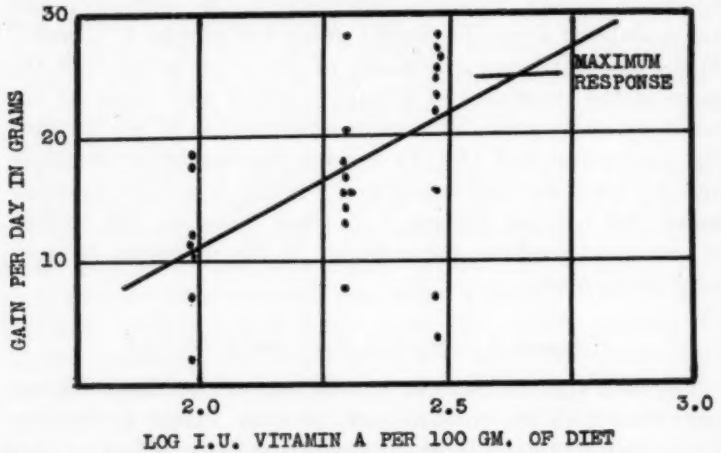

Fig. 1 Scatter diagram showing the gain per day for each poult during the third and 4th weeks. Regression line calculated from weight data of groups 1, 2, and 3 during this period of the experiment.

groups 4, 5, 6, and 7 were sacrificed because they suffered from perosis but there were no deaths in these groups due to a deficiency of vitamin A.

Post mortem examination revealed a high incidence of nodules on the mucous membrane of the lower esophagus and the crop of poults in groups 1 and 8. One bird in group 2 and one in group 3 which died also showed small nodules of the crop. Lesions of the upper esophagus were infrequent, confirming the observations reported by Hinshaw and Lloyd

('34). No evidence of an accumulation of urates in the ureters or kidneys was noted. This condition differs markedly from the characteristic whitish appearance of the kidneys of vitamin A-deficient chicks. Hinshaw and Lloyd ('34) also reported no excessive accumulation of urates in vitamin A-deficient turkey poults fed a ration consisting of natural feedstuffs.

The data indicate that the vitamin A requirement of young poults for the prevention of all signs of deficiency is in excess of 400 I.U. per 100 gm of diet. Since no signs of deficiency were observed in group 5, a vitamin A level of 500 I.U. per 100 gm of diet appears to be adequate.

Liver storage of vitamin A

No vitamin A was found in the livers of the poults which died during the course of the experiment. At the termination of the investigation, the birds of group 6 (600 I.U. of vitamin A per 100 gm of diet) showed only a trace of liver vitamin A, whereas group 7 (turkey starting mash) showed an average of 100 I.U. of vitamin A per liver. No liver storage of vitamin A was found in the other groups.

Rubin and Bird ('41) have shown that the liver stores of vitamin A in chicks fed a deficient diet show a decrease which is even greater than that evidenced during starvation. In further studies with chicks these workers found that, during the first 5 weeks of life, the vitamin A stores increased only slightly even with a good ration. In the present experiment, the poults were fed a diet devoid of vitamin A in a preliminary depletion period of 9 days. The loss of vitamin A during this period may account for the absence of liver storage after 6 weeks on experiment, since the highest level fed was only in slight excess of the requirement for growth and normal development. Under the conditions of this experiment, a vitamin A level in excess of 600 I.U. per 100 gm of ration appears to be necessary to promote liver storage of the factor.

GENERAL DISCUSSION

The vitamin A requirement of Jersey Buff turkey poults fed a purified diet was found to be somewhat lower for growth than for the prevention of other signs of vitamin A deficiency. A diet containing 300 I.U. per 100 gm promoted almost maximum growth, whereas 500 I.U. were required to prevent entirely unsteadiness of gait, ruffled feathers, and an abnormal condition of the inner eyelid.

Although Taylor, Stern, Russell and Jungherr ('47) have reported that the nasal histopathology of chicks showed signs of borderline vitamin A deficiency at levels much above that required to prevent all other signs of the deficiency, these findings have not been considered important from the practical standpoint. Such work was therefore not attempted in the present studies. Scott, Jungherr and Matterson (personal communication), using cod liver oil and cereal grass as sources of vitamin A, have observed suspicious nasal lesions in young turkeys at levels as high as 750 I.U. per 100 gm of ration, whereas no lesions were observed at 1,000 I.U. of vitamin A. For the prevention of the gross signs of deficiency in growing poults, 500 I.U. of vitamin A per 100 gm of diet (2,250 I.U. per lb.) appear adequate.

Wharton and associates ('49) have recently reported the requirement for growing turkeys to be 1,700 I.U. per 100 gm of feed, a level over three times that arrived at in our experiment and by other workers. The reasons for the difference are not evident. The experimental plan was essentially the same for the two experiments but, as might be expected, they differed in details of execution. The principal differences were that Wharton and associates used a heavy breed, the Broad Breasted Bronze strain, and cod liver oil as a source of vitamin A, whereas we used a breed of lighter weight, the Jersey Buff, and a concentrate of the vitamin in natural ester form. In our experiment a 9-day depletion period was used but it is not likely that this would ultimately affect maximum growth. At a level of 500 I.U. per 100 gm of feed in our experiment,

liver storage of the vitamin did not occur. This level was adequate, however, for growth and the prevention of all gross signs of deficiency, which suggests that liver storage is not necessary to produce a normal bird. There is the possibility that the discrepancy is due to a difference in breed requirements.

SUMMARY

When Jersey Buff turkey poults were fed a purified diet containing graded levels of vitamin A, 300 I.U. per 100 gm gave nearly as good growth as 500 I.U. or 600 I.U., but a level of 500 I.U. was necessary to prevent gross external and internal signs of A-avitaminosis. It is recognized that higher levels of vitamin A may be necessary for the prevention of microscopic signs of deficiency.

ACKNOWLEDGMENTS

We are indebted to E. I. duPont de Nemours and Co., New Brunswick, N. J., for the supply of activated animal sterol (Delsterol) and to Distillation Products, Inc., Rochester, N. Y., for vitamin A concentrate in natural ester form (Myva-Dry Powder).

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EFFECTS OF DIFFERENT AMOUNTS OF LUTEIN, SQUALENE, PHYTOL AND RELATED SUBSTANCES ON THE UTILIZATION OF CAROTENE AND VITAMIN A FOR STORAGE AND GROWTH IN THE RAT [1]

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ONE FIGURE

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Relatively large amounts of lutein impair the utilization of β-carotene (Kemmerer et al., '47; Kelley and Day, '48a; Johnson and Baumann, '48a). However, small amounts of xanthophyll appear to promote carotene utilization, as is indicated by the effect on weight gain and mortality when the intake of the provitamin is low (Sherman, '47). These seemingly contradictory findings suggested the need for a study of the effect of the carotene: lutein ratio on the utilizability of carotene. Also it was of interest to investigate the effects of various other structurally related compounds on the utilization of carotene and vitamin A. As has been pointed out by John-

[1] Supported in part by grants from the Nutrition Foundation, Inc., and the Research Funds of the Graduate School of Indiana University. Some of the data are taken from a thesis submitted by Edward G. High to the Faculty of the Graduate School in partial fulfillment of the requirements for the Ph.D. degree, December, 1949.

Presented in part at the meetings of the American Chemical Society in Houston, March, 1950.

Contribution no. 510 from the Department of Chemistry, Indiana University, Bloomington.

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son and Baumann ('48a) and by Kelley and Day ('50), there is reason to suspect that some of these compounds are antagonists of vitamin A, or that they impair the utilization of its precursors. No true biochemical antagonist of vitamin A has been thoroughly demonstrated but an oxidation product of the vitamin appears to have this property (Meunier et al., '50).

EXPERIMENTAL

The lutein used was prepared from concentrates.[3] The procedure employed was that of Zechmeister and Sease ('43) modified for use with these concentrates. The materials were saponified with concentrated methanolic potassium hydroxide in a low actinic container. The saponified material was extracted with two portions of peroxide-free ether and the combined extracts were washed in a continuous washing apparatus (LeRosen, '42) and dried over anhydrous sodium sulfate. After removing the solvent under vacuum, the crude lutein was dissolved in pure benzene at 40°C. and precipitated by adding three volumes of purified Skelly-solve B. The precipitate was filtered under nitrogen and redissolved in a minimum amount of chloroform. From this it was reprecipitated with 4 volumes of Skelly-solve B. Finally the filtered crystals were dried in a vacuum over phosphorus pentoxide and paraffin. This preparation, dissolved in 95% twice-distilled ethanol, possessed characteristic absorption maxima of 422, 445, and 472–474 mμ as determined in a Beckman spectrophotometer.

The required amount of carotene[4] (85% β- and 15% α-carotene), menthol, and hydroquinone were dissolved in peroxide-free ether and mixed with a determined amount of cottonseed oil[5] to give the desired concentration in 0.2 ml of the oil. The ether in each was removed under reduced

[3] Obtained from the Midwest Extraction Co. Rockford, Ill., and the American Chlorophyll Co., Alexandria, Va. (now Lakeland, Fla.).

[4] Obtained from General Biochemicals, Inc., Chagrin Falls, O.

[5] Wesson.

pressure. The vitamin A concentrate,[6] squalene,[7] phytol,[8] α-tocopheryl acetate,[9] geraniol,[10] methyl ionone,[11] α-ionone,[12] and β-ionone [13] were dissolved directly in a determined amount of cottonseed oil to give the desired concentration in 0.2 ml of the oil. All the supplements were stored at 4°C. The light sensitive substances were kept under nitrogen in low actinic flasks. The supplements were administered substantially in the same way as that described by Kelley and Day ('50). Similarly, the Kelley and Day ('50) method was used to deprive the rats of their vitamin A stores. All the animals were maintained in air conditioned quarters at 25°C.

Approximately 16 hours after the administration of the last supplement each rat was sacrificed with ether, except in the case of the rats used to determine the effect of repeated doses of ether on the disappearance of vitamin A from the tissues. The latter were killed by direct decapitation. The livers and kidneys were removed promptly and analyzed for vitamin A by the colorimetric method of Sobel and Werbin ('46), the procedure being the same as that used previously by Kelley and Day ('48b).

Effect of β-carotene: lutein ratio on the deposition of vitamin A in the tissues

Six series of vitamin A-depleted rats were used to determine the effect of different amounts of lutein on the deposition of vitamin A in the liver and kidneys, with the total daily

[6] Containing approximately 200,000 U.S.P. units per gram. Kindly supplied by Distillation Products, Inc., Rochester, N. Y.

[7] Produced by molecular distillation from liver oils of basking shark and containing more than 90% squalene. Furnished by Distillation Products, Inc., through the courtesy of Dr. Norris Embree.

[8] Obtained from the American Chlorophyll Co., Alexandria, Va. (now Lakeland, Fla.).

[9] Obtained from Merck and Co., Rahway, N. J.

[10] Obtained from Eimer and Amend Co., N. Y.

[11] Obtained from the Verona Co., N. Y.

[12] Obtained from Dodge and Olcott Co., N. Y.

[13] See footnote 12.

carotene supplement ranging from 15 μg to 60 μg. The carotene: lutein ratios were from 0.03 to 3. Although the liver and kidneys were analyzed separately, only the sum of the values for each rat is reported. The data are summarized in table 1. In rats given carotene alone, the total vitamin A in the tissues increased, as was expected, with increasing amounts of carotene. However, at each level of carotene the total vitamin A deposition was higher when the carotene: lutein ratio was 1.2 or greater, but the content of vitamin A was markedly decreased when the ratio was as low as 0.06. For example, the total vitamin A was 8.5 μg when 15 μg of carotene alone were fed daily. In the same series 5 μg of lutein and 15 μg of carotene per rat daily resulted in the deposition of 11.7 μg of vitamin A. At the level of 50 μg of lutein per day, besides 15 μg of carotene, the vitamin A deposition was only 6.3 μg; and when 500 μg of lutein were administered each day with the carotene, the amount of vitamin A was even less, viz., 5.2 μg.

At an intake level of 30 μg of carotene and 500 μg of lutein per day the amount of tissue vitamin A was 32% less than in animals given the carotene alone (series 3). However, at a carotene: lutein ratio of 1.2, viz., 30: 25, the tissues contained 53% more vitamin A. In other words, increasing the lutein from 25 μg to 500 μg per day without changing the intake of carotene resulted in a decrease of over 100% in the amount of vitamin A deposited. These data are representative of others that were obtained at different levels of carotene and lutein. Also, the same effects were found in another strain of rats (series 4 and 6, table 1). In no case did the level of lutein administered seem to affect the growth rate.

Effect of α- and β-ionones and squalene on the utilization of carotene and vitamin A

Having established that lutein antagonizes the storage of vitamin A from carotene only when the carotene: lutein ratio is low, it was of interest to determine the effect of other

TABLE 1

Effect of β-carotene (C): lutein (L) ration on deposition of vitamin A in tissues of rats

SERIES	DAILY SUPPLEMENT	DAYS ON SUPPLEMENT	NO. AND SEX OF RATS	C:L RATIO	WT. GAIN	TOTAL VITAMIN A (LIVER AND KIDNEYS)
					gm	*μg*
1	a. 15 μg C	20 to 24	14 M		67	8.5 + 1.2 [1]
	b. same plus 5 μg L	20 to 24	4 M	3	66	11.7 + 0.7
	c. same as a. plus 50 μg L	20 to 24	4 M	0.3	52	6.3 + 0.8
	d. same as a. plus 500 μg L	20 to 24	5 M	0.03	61	5.2 + 1.3
2	a. 20 μg C	23 to 25	6 F		49	21.7 + 2.9
	b. same plus 50 μg L	23	3 F	0.4	53	22.9 + 7.9
	c. same as a. plus 500 μg L	25	4 F	0.04	38	16.5 + 3.9
3	a. 30 μg C	18	6 (5 M)		86	15.7 + 3.1
	b. same plus 25 μg L	18	6 (4 M)	1.2	78	24.0 + 3.5
	c. same as a. plus 500 μg L	18	7 (5 M)	0.06	80	10.8 + 0.9
4 [2]	a. 30 μg C	18	3 F		28	39.3 + 4.5
	b. same plus 25 μg L	18	3 F	1.2	35	58.5 + 4.3
	c. same as a. plus 500 μg L	18	3 F	0.06	42	28.4 + 6.7
5	a. 60 μg C	19	4 F		61	87.7 + 19.0
	b. same plus 25 μg L	19	4 F	2.4	47	100.8 + 14.0
	c. same as a. plus 500 μg L	19	4 F	0.12	54	74.6 + 14.6
6 [2]	a. 60 μg C	19	3 M		80	52.5 + 10.2
	b. same plus 25 μg L	19	3 M	2.4	67	73.3 + 7.8
	c. same as a. plus 500 μg L	19	3 M	0.12	69	71.0 + 2.5

[1] The standard error of the mean $= \sqrt{\frac{(X-x)^2}{n(n-1)}}$ X = individual values, x = the group mean, and n = the number of rats per group.

[2] McCollum strain rats.

isoprenes with the carotene or vitamin A intake at relatively low levels. Some of the results from these investigations are summarized in table 2. α-Ionone and β-ionone were each tested at the level of 500 μg per day for 12 days on rats given 30 μg of β-carotene in one series and 13 μg of vitamin A in a second series. The compounds had no effect on the amount of vitamin A deposited in either the liver or the kidneys. Similarly, there was no effect on the growth response to the carotene or vitamin A. The lack of response to the α-ionone is not in harmony with the plausible suggestion of Johnson and Baumann ('48a) that "the low storage-promoting power of α-carotene may be a specific inhibitory influence of the α-ionone ring, a possibility suggested by the fact that lutein minimizes vitamin A storage due to β-carotene."

Squalene in large amounts had a marked effect. For example, in rats given 30 μg of β-carotene and 10 mg of squalene daily for 12 to 14 days the total vitamin A found in the liver and kidneys was 10.7 μg, as compared with 17.8 μg for controls given carotene alone. This difference was considerably greater in each of three other series fed 50 mg of squalene daily in addition to 30 μg of β-carotene. At the level of 10 mg per rat daily squalene affected mainly the content of vitamin A in the liver; whereas at the level of 50 mg there was a marked diminution in both liver and kidney vitamin A. Unlike lutein (Kelley and Day, '50), squalene did not impair the utilization of vitamin A. This was established by the use of two different series involving 26 rats. In one series the rats were given 13 μg of vitamin A and 50 mg of squalene per day for 13 days. In the other series the same amounts were fed for 15 days. The squalene had no effect on the growth of any of the rats.

Effect of α-tocopheryl acetate, phytol and hydroquinone on the utilizaton of carotene and vitamin A

Even though squalene impaired the utilization of β-carotene, the failure of this non-oxyisoprenic compound to affect

TABLE 2

Deposition of vitamin A in rats fed α-ionone, β-ionone, or squalene along with β-carotene (C) or vitamin A (A)

SERIES	COMPOUNDS	DAYS FED	NO. AND SEX OF RATS	WT. GAIN	TOTAL VITAMIN A (LIVER AND KIDNEYS)
				gm	*μg*
1	a. 30 μg C	12	8 (2 M)	26	24.3 + 3.0 [1]
	b. same plus 500 μg α-ionone	12	8 (3 M)	35	20.4 + 2.2
	c. same as a. plus 500 μg β-ionone	12	10 (3 M)	28	20.2 + 1.9
2	a. 12 μg A	12	7 (4 M)	32	27.7 + 1.9
	b. same plus 500 μg α-ionone	12	7 (4 M)	28	28.9 + 2.4
	c. same as a. plus 500 μg β-ionone	12	7 (4 M)	34	28.4 + 2.2
3	a. 30 μg C	12–14	9 (3 M)	47	17.8 + 1.6
	b. same plus 10 mg squalene	12–14	8 (2 M)	44	10.7 + 1.5
4	a. 30 μg C	19	9 (3 M)	44	25.9 + 3.7
	b. same plus 50 mg squalene	19	9 (3 M)	47	6.8 + 1.0
5	a. 30 μg C	18	5 M	58	15.9 + 2.8
	b. same plus 50 mg squalene	18	5 M	56	5.8 + 1.3
6	a. 30 μg C	21	4 (3 M)	54	19.3 + 3.7
	b. same plus 50 mg squalene	21	4 (3 M)	58	4.8 + 0.9
7	a. 13 μg A	13	9 (6 M)	41	39.8 + 2.9
	b. same plus 50 mg squalene	13	8 (5 M)	52	38.8 + 2.5
8	a. 13 μg A	15	5 (3 M)	34	44.6 + 1.6
	b. same plus 50 mg squalene	15	4 (3 M)	34	43.9 + 1.0

[1] Standard error of the mean.

the utilization of preformed vitamin A indicated the value of investigating phytol and α-tocopherol. The latter are oxyisoprenic compounds and in that respect have some resemblance to vitamin A. By the time this phase of the work was initiated, Johnson and Baumann ('48b) had reported the inhibitory effect of α-tocopherol on the utilization of carotene and the lack of inhibition on vitamin A utilization.

As is shown in table 3, series 1, 10 mg of α-tocopheryl acetate per rat daily for 13 to 16 days definitely impaired the utilization of carotene when the level of the latter was 30 μg per day. Thus the findings of Johnson and Baumann ('48b) in this regard were confirmed. It was logical to consider that the inhibiting effect of the tocopheryl acetate might be due to the phytol moiety. Rats fed a stoichometric amount of phytol (6 mg) daily showed no impairment in the utilization of carotene. However, in animals given the same amount of carotene but 50 mg of phytol daily, the vitamin A deposition was certainly decreased. In another series (series 3, table 3) the carotene and phytol were fed at the levels of 26 μg and 40 mg, respectively. Here also the phytol decreased the vitamin A storage. The kidneys of phytol-fed rats seemed to show a greater decrease than the livers. In addition, the rats given large amounts of phytol seemed to gain weight at a lower rate than animals receiving carotene alone. This may have been due to some effect unrelated to the metabolism of carotene and vitamin A. In general it is found that vitamin A-depleted rats which are forced to gain at slower rates deposit larger amounts of vitamin A when supplemented with carotene or vitamin A (Johnson and Baumann, '48c). Consequently, the vitamin A storage might have been even lower had it been possible for the phytol-fed rats to gain weight at the rate of their controls.

The storage of vitamin A was not affected by 50 mg of phytol when the vitamin-depleted rats were supplemented with 13 μg or 7 μg of vitamin A; neither was there any effect on the rate of growth. Thus the effect of phytol was qualita-

TABLE 3

Effects of α-tocopheryl acetate, phytol and hydroquinone on the deposition of vitamin A in rats following supplementation with β-carotene (C) or vitamin A (A)

SERIES	COMPOUNDS FED	DAYS FED	NO. AND SEX OF RATS	WT. GAIN	TOTAL VITAMIN A (LIVER AND KIDNEYS)
				gm	*μg*
1	a. 30 μg C	13–16	7 (3 M)	53	14.5 + 2.6[1]
	b. same plus 10 mg α-tocopheryl acetate	13–16	6 (4 M)	55	7.4 + 0.8
	c. same as a. plus 6 mg phytol	13–16	6 (4 M)	51	15.8 + 2.8
2	a. 30 μg C	13	9 (7 M)	30	19.3 + 1.7
	b. same plus 50 mg phytol	13	8 (6 M)	15	12.4 + 1.7
3	a. 26 μg C	13	5 (3 M)	47	15.6 + 1.9
	b. same plus 40 mg phytol	13	4 (2 M)	26	10.1 + 0.6
4	a. 13 μg A	13	4 (3 M)	34	36.6 + 2.6
	b. same plus 50 mg phytol	13	4 (3 M)	28	38.8 + 3.7
5	a. 7 μg A	14	5 (3 M)	54	14.2 + 1.0
	b. same plus 50 mg phytol	14	5 (3 M)	51	16.2 + 1.7
6	a. 30 μg C	13	6 (4 M)	33	19.7 + 2.5
	b. same plus 5 mg hydroquinone	13	6 (4 M)	32	20.1 + 4.4
	c. same as a. plus 1 mg hydroquinone	13	6 (4 M)	42	23.8 + 2.6
7	a. 30 μg C	19–20	6 (3 M)	56	24.9 + 4.7
	b. same plus 5 mg hydroquinone	19–20	6 (3 M)	59	25.5 + 3.7
	c. same as a. plus 1 mg hydroquinone	19–20	6 (3 M)	60	22.1 + 2.8

[1] Standard error of the mean.

tively similar to that of squalene, except that there was no indication of growth impairment by the latter.

Owing to the anti-oxidant properties of α-tocopheryl acetate as well as the free alcohol, *in vivo,* it seemed possible that the effect of large amounts of the vitamin might be due to a non-specific impairment of oxidative processes in the utilization of carotene. Hydroquinone was chosen to test this possibility, although its solubility and structure are quite different from the chroman moiety of α-tocopherol. The compound was fed with 30 μg of carotene at levels of 5 mg and 1 mg per rat daily in two different series. In neither case was there any effect on carotene utilization or on the rate of growth. Hickman et al. ('44) also noted that hydroquinone fails to protect either carotene or vitamin A from destruction *in vivo.*

Effects of various substances on the rate of depletion of vitamin A stores

Because squalene, as well as a few other isoprenic compounds, inhibited the utilization of carotene, it was of interest to determine the effect of this substance and others on the rate of disappearance of vitamin A from the tissues. The results and the methods used are given in table 4. Rats containing considerable amounts of vitamin A were fed 50 mg of squalene daily for 14 days. The total vitamin A stores at the end of this time were substantially the same as those of controls which received no squalene. Similarly, other series were given phytol, methyl ionone, geraniol and menthol. In no case was there any effect on the rate of decrease in the content of tissue vitamin A.

Since it had been reported that ether hastens the depletion of hepatic vitamin A (Chevallier and Choron, '35) rats were subjected to light ether anesthesia for approximately three minutes daily for 9 days. This did not change the amount of vitamin A in either the liver or the kidneys, as is shown in table 4.

TABLE 4

Effect of various substances on the rate of depletion of tissue vitamin A in rats. (Two to three days before beginning the administration of the test substances each rat was given three to 6 oral doses of a vitamin A concentrate. Those in series 1 and 2 received 1,650 μg each; those in series 3, 1,100 μg; and those in series 4, 825 μg)

SERIES		SUBSTANCES	DAYS GIVEN	NO. AND SEX OF RATS	WT. GAIN	TOTAL VITAMIN A (LIVER AND KIDNEYS)
					gm	*μg*
1	a.	0.2 ml cottonseed oil [1]	14	4 F	3	1,004 + 207 [2]
	b.	same plus 50 mg squalene	14	5 F	4	1,055 + 173
2	a.	0.2 ml cottonseed oil	11	5 (2 M)	24	980 + 155
	b.	same plus 20 mg phytol	11	5 (2 M)	23	945 + 64
3	a.	0.2 ml cottonseed oil	9 to 17	11 F	13	625 + 15
	b.	same plus 50 gm geraniol	9 to 17	11 F	6	620 + 20
4	a.	0.2 ml cottonseed oil	8 to 11	12 (9 M)	21	530 + 26
	b.	same plus 50 mg methyl ionone	8 to 11	4 (2 M)	8	570 + 16
	c.	same as a. plus 50 mg geraniol	8 to 11	4 (2 M)	13	467 + 31
	d.	same as a. plus 50 mg menthol	8 to 11	4 (2 M)	14	451 + 87
5	a.	control	9	5 (3 M)	24	385 + 31
	b.	ether to light anesthesia	9	4 (3 M)	19	350 + 23

[1] Wesson oil.
[2] Standard error of the mean.

Effect of growth rate on liver and kidney vitamin A

The data accumulated in this study have some bearing on the difference between males and females with regard to the storage of vitamin A. Several investigators have been concerned with this question. Brenner et al. ('42) concluded that there is a significant sex difference, but Lemley et al. ('47) found no difference.

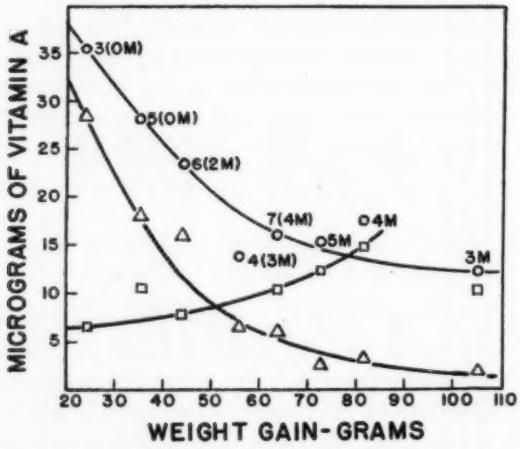

Fig. 1 Relation between rate of growth and the liver and kidney vitamin A content when 30 μg of carotene were fed daily. △ = hepatic vitamin A; □ = kidney vitamin A; and ○ = sum of hepatic and kidney vitamin A. The number and sex of the rats in each weight group are indicated beside the points on the upper curve.

Control vitamin A-depleted rats that received 30 μg of β-carotene alone per rat per day for approximately 18 days were divided into 8 groups on the basis of weight gains subsequent to the beginning of the supplements. The final difference in average weight gains between each group was 10 to 15 gm. The average weight gain of each group was plotted against the average content of vitamin A in the liver and in the kidneys. The results are illustrated in figure 1. As is

shown, the amount of the vitamin in the kidneys tends to increase as a function of the rate of growth. Conversely, the hepatic vitamin A decreases as the rate of growth increases. The general pattern of these relationships was found in animals supplemented with different amounts of carotene or vitamin A. The results are in line with the data of Johnson and Baumann ('48c), who showed that restriction in the growth rate promotes the retention of vitamin A in the liver.

Although most of the females had relatively more vitamin A than males subjected to the same experimental conditions, there seemed to be no sex differences aside from the difference in rate of growth.

DISCUSSION

Sufficient information is not yet available to explain the effects of lutein, squalene, phytol and α-tocopherol on the utilization of carotene. None of these materials in large amounts, except lutein and possibly phytol, seems to have any adverse effect on the utilization of preformed vitamin A for the deposition of this vitamin in the liver and kidneys. In large amounts lutein appears to inhibit fed vitamin A as much as fed β-carotene (Kelley and Day, '50). However lutein, as well as squalene, phytol, α-ionone, β-ionone, and geraniol, has no effect on the rate of disappearance of vitamin A from the liver. This is evidence that the alimentary tract is the site of action of all the isoprenic substances demonstrated to have any effect on the utilization of carotene or vitamin A. Lutein as such would have to exert its effect in the alimentary tract because it is not absorbed in detectable amounts by the rat. The possibility must be considered, of course, that some lutein may be cleaved symmetrically into two hydroxy ionones (α- and β-), perhaps by the enzyme system that converts β-carotene to vitamin A. Neither of these hypothetical degradation products should be expected to have any vitamin A activity, but one or both might impair the deposition of vitamin A in the tissues, possibly in a competitive manner. Thus the unique effect of large amounts of

lutein in inhibiting the utilization of vitamin A, as well as β-carotene, may be due to such a metabolic pattern.

On the other hand, small amounts of lutein, as demonstrated here, and α-tocopherol at low levels (Harris et al., '44) promote the utilization of carotene. There is no ready explanation of these seemingly contradictory facts. In this case it would seem that the action of lutein may be analogous to that of the tocopherols in the oxidation of fats. As was reported by Swift ('48), tocopherols efficiently promote an anti-oxidant effect on fats at low levels, but with increasing amounts of tocopherols the efficiency progressively diminishes until at quite high concentrations the stability of test fats is actually decreased. Data on the anti-oxidant effect of lutein are not at hand, but if it should be found to be qualitatively analogous to the tocopherols this would seem to explain the results on lutein presented here.

Apparently none of the other isoprenic substances considered here, except α-tocopherol, has been investigated in regard to its effects on the utilization of carotene or vitamin A. In particular squalene (Channon and Tristram, '37) and phytol (Channon and Collinson, '28) have been shown to be absorbed by the rat. Both are widely distributed in foods, the former in various vegetable oils as well as in animal fats (Fitelson, '43). The occurrence of phytol in chlorophyll is well known. The amounts normally ingested certainly are not high enough to impair the utilization of carotene. Investigations now in progress are designed to reveal more information on the effect of these substances of carotene and vitamin A.

SUMMARY

Vitamin A-depleted weanling rats were fed different levels of β-carotene. The addition of small amounts of lutein increased the quantity of vitamin A stored in the livers and kidneys but large amounts decreased the storage. Also, large amounts of squalene, phytol and α-tocopheryl acetate decreased the storage of vitamin A. Relatively small amounts of α-ionone, β-ionone, and hydroquinone had no effect.

The storage of vitamin A given to vitamin A-depleted rats was not affected by large supplements of squalene or phytol.

The rate of loss of vitamin A from the livers and kidneys of rats was not affected by any of the following supplements: squalene, phytol, geraniol, menthol, or by repeated anesthesia with ethyl ether.

The sex difference in the utilization of carotene and vitamin A for storage of vitamin A in growing rats is due, in large part at least, to the differences in rate of growth.

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EVALUATION BY DOG GROWTH OF EGG YOLK PROTEIN AND SIX OTHER PARTIALLY PURIFIED PROTEINS, SOME AFTER HEAT TREATMENT

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FOUR FIGURES

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Earlier studies have shown that dry heat treatment of proteins at temperatures over 120° decreases their growth-promoting qualities (Morgan, '31). Greaves, Morgan and Loveen ('38) reported that the growth-promoting value of dry crude casein for rats was impaired by heating at 140° for 30 minutes. They found that supplementation with 0.2% lysine monohydrochloride largely overcame the deficiency. Injury of lysine due to heating proteins was also observed by Eldred and Rodney ('46), Block et al. ('34, '46), Waisman and Elvehjem ('38) and Devlin and Zittle ('44). All of these growth studies were conducted with rats. Weast, Groody and Morgan ('48) found that heating a crude casein from 140° to 200° considerably impaired its growth value for dogs.

This study was undertaken chiefly to compare the effects of the heating of a purified vitamin-free casein and a defatted whole egg powder on their growth-promoting ability in young dogs. Egg albumin, wheat gluten, peanut meal, dried beef muscle and raw egg yolk were also assayed. The growth of young dogs was used as the principal measure of biological value and the results are expressed in terms of the protein indexes.

METHODS

All of the isocaloric diets used in this experiment except that containing egg yolk were identical in composition except for the source and level of the protein. The egg protein diets were fed at a level of 12% in order to obtain approximately the same amount of growth that had been obtained with the 18% casein diets. Earlier experiments had revealed that diets containing less than 18% casein did not allow even subnormal growth, since the dogs refused to eat the diets. All the proteins, with the exception of the raw egg yolk, were supplied by the Bureau of Biological Research of Rutgers University as part of a cooperative project engaged in by several laboratories.

The preparation of the 6 protein sources is described in full in an addendum to the article by Hawley, Murlin, Nasset and Szymanski ('48) describing their study of the use of these protein foods by adult human subjects.

The nitrogen contents, in per cents of the foods as determined in this laboratory, were as follows: casein 14.12, peanut flour 9.25, egg albumin 11.80, whole egg powder 11.80, dried beef muscle 14.41, gluten 12.95.

The casein for two of the diets was heated according to the method previously described (Weast et al., '48); that is, it was heated for 15 minutes in layers two to two and one-half inches deep at 140°. In one diet this heated casein was supplemented with 0.6% L (+) — monohydrochloride lysine.[1] To another diet containing the unheated vitamin-free casein was added 1.0% DL-methionine.

The defatted powdered whole egg was heated in the same manner as the casein. It was found to be much more difficult to control the heat treatment of the egg powder than of the casein.

Fresh raw egg yolk was used as a source of protein in one of the diets. Nitrogen was determined by the macro-Kjeldahl method on each fresh lot of yolk. The yolk was diluted

[1] The lysine was supplied by the Interchemical Corporation, Biochemical Division, Union, New Jersey, through the courtesy of Dr. M. G. Mulinos.

with water in the ratio of one to one and fed with a basal diet consisting of salts, $CaCO_3$, vitamin mix and sugar to make it isocaloric with the other diets. The fat and $CaCO_3$ contents of this diet were different from those of the other diets since the amount of $CaCO_3$ was increased in order to maintain the Ca:P ratio at two to one and since the egg yolk itself contained 33% fat, hence no fat was added as such.

All the diets except that containing raw egg yolk were made up as follows, in per cent: protein 12, 18 or 40, salts 5 [2] 2.5, $CaCO_3$ 1.5, fat [3] 10, and sucrose sufficient to provide 430 cal. per 100 gm of diet. The egg yolk diet contained fat 26.0, protein 12.0, salts 5 2.5, $CaCO_3$ 1.86, sucrose 36. The peanut flour was found to contain 5.75% fat and this was allowed for in calculating the amount of fat added to that diet.

All the necessary vitamins were provided in equal amounts in all diets. The amounts given, in milligrams per kilogram per day, were as follows: thiamine, riboflavin, pyridoxine and folic acid, each 0.12, calcium pantothenate and para-aminobenzoic acid, each 3.6, nicotinamide 2.4, inositol 30, choline 60, biotin 0.006. Vitamins A and D were given separately so as to supply 400 I.U. of vitamin A and 50 I.U. of vitamin D per kilogram per day.

Thirty-seven purebred cocker spaniels from 8 litters were used and were kept in individual standard metabolism cages. When they weighed about 1.5 kg at 5 to 7 weeks of age they were started on the diets. These were offered at first at the level of 60 gm, 260 cal., daily per kilogram body weight, and any uneaten portions were weighed. The intakes of all animals were kept, so far as possible, at the level of the animal which ate the least, although exact equalization of intake of young dogs is not possible without forced feeding. Exact records of intake were made daily. The feeding periods ranged from 120 to 188 days.

[2] Axelrod, H. E., A. F. Morgan and S. Lepkovsky ('45).

[3] Primex, hydrogenated vegetable fat.

Blood analyses were done once a month after the dogs had been on the diets about two months. Total serum protein, serum albumin, hemoglobin and packed cell volumes were determined, and red blood cell counts were made. Albumin-globulin fractionation was done by the method of Kingsley ('40). Total protein and albumin were determined by the micro-Kjeldahl method. Hemoglobin was determined by the method of Newcomer ('23), packed cell volume by the use of the Wintrobe tube.

Five-day N balance studies were made on most of the dogs on 5 or 6 occasions. The nitrogen content of pooled 5-day fecal collections was measured by the macro-Kjeldahl method, that of urine collections by micro-Kjeldahl. Urine was collected under toluene and sulfuric acid and refrigerated. The feces were dried daily. Since diarrhea sometimes made collections difficult, cellu flour was fed two or three days preceding as well as during the collections. Sulfaguanidine was also given to some of the dogs for a short time, but due to unfavorable findings which were apparently attributable to the drug, this form of therapy was withdrawn.

Creatine determinations on fresh heart and muscle tissue of dogs on the casein and egg diets were made on autopsy by the method of Rose, Helmer and Chanutin ('27) modified for use with the Klett colorimeter. Nitrogen and moisture were determined in liver, heart, kidney and muscles at autopsy.

RESULTS AND DISCUSSION

Protein indexes

The growth per gram of nitrogen intake was calculated separately for 30, 60, 90, 120 and the total number of days the dogs were fed the diets. The period chosen for comparison was 120 days, since the most consistent values as well as the major part of the rapid growth appeared here.

The total gains in weight in kilograms in 120 days made by the dogs, disregarding intake, put the proteins in the following order: beef 7.8, casein 5.5, gluten at 40% level 4.7,

egg yolk 4.5, casein plus methionine 4.0, defatted whole egg 3.5, heated egg and peanut flour, each 2.1, gluten at 18% level and egg albumin at both levels, 1.1 (table 1). The data concerning blood composition (table 3), and the rate of nitrogen retention (fig. 4), as well as the observations of the condition of the organs at autopsy and the general appearance of the hair, eyes, muscle turgor and activity of the animals, do not confirm this order of values. The gain per gram of nitrogen intake offers a more consistent guide.

The average values for protein efficiency are shown in table 1 along with the standard errors. It is realized that the number of dogs in each group is small and that the validity of a statistical treatment of the data may therefore be questionable. The figures are offered, however, in order to portray the encountered variability. The difficulty and expense involved in this type of experiment with dogs are large as compared with similar experiments with small animals. The individual variation among the dogs of this colony compares favorably, however, with that seen in rat colonies.

The protein efficiency of the raw egg yolk, 12.7 ± 0.8, was clearly superior to that of all the proteins studied. The defatted whole egg, beef muscle and unheated casein were alike and were superior to the remaining proteins, although the difference between these and the heated casein both with and without lysine was possibly not significant. The inferiority of the heated egg, peanut flour, gluten and egg albumin was evident.

If egg yolk, which produced the greatest gain per gram nitrogen eaten, is given the assumed value of 100, the other proteins can be given an index number relative to this (table 2). The beef, unheated egg powder and casein had values of about 75. Heating the whole egg powder reduced its growth-producing quality to 63. Heat treatment of the casein did not cause comparable damage, nor did the addition of 0.6% L-lysine to the heated casein produce much change. The addition of 1.0% DL-methionine to the unheated casein apparently depressed the growth-promoting value from 74

TABLE 1

Efficiency of egg yolk and 6 partially purified proteins for the growth of dogs in 120 days

PROTEIN	DIETARY LEVEL	DOG NO. AND SEX	BODY WEIGHTS			LITTER NUMBER	TOTAL N INTAKE	GAIN/GM N INTAKE	AVE. ± S.E.[4]
			at beginning	at end	gain				
	%		*kg*	*kg*	*kg*		*gm*	*gm*	
Unheated casein	18	1 ♂	1.7	6.4	4.7	1	504	9.3	9.4 ± 0.3
		5 ♀	1.5	5.9	4.4	2	451	9.8	
		7 ♂	2.2	8.7	6.5	3	730	9.0	
Unheated casein + 1% methionine	18	10 ♂	1.9	5.1	3.2	4	533	6.0	7.3
		11 ♀	1.7	6.6	4.9	4	576	8.7	
Heated casein	18	2 ♂	1.5	6.3	4.8	1	510	9.4	8.7 ± 0.5
		6 ♀	1.6	5.7	4.1	2	461	8.9	
		8 ♀	1.6	6.1	4.5	3	570	7.9	
Heated casein + 0.6% lysine	18	3 ♂	1.5	5.6	4.1	1	450	9.1	8.9 ± 0.4
		4 ♀	1.5	5.7	4.2	1	450	9.3	
		9 ♂	2.2	7.6	5.4	4	658	8.2	
Defatted dried whole egg	12	12 ♂	2.4	7.3	4.9	4	458	10.7	9.6 ± 0.7
		13 ♀	1.7	5.8	4.1	4	402	10.2	
		16 ♂	1.8	4.8	3.0	5	298	10.1	
		23 ♂	1.7	3.8	2.1	6	278	7.5	
Heated dried whole egg	12	14 ♀	2.7	5.0	2.2	4	276	8.0	8.1 ± 0.8
		15 ♂	1.7	3.7	2.0	4	228	8.7	
		20 ♂	1.8	1.9	0.1	5	177	0.6[1]	
		21 ♂	1.8	3.9	2.1	6	280	7.5	
Raw egg yolk	12	17 ♀	1.5	5.4	3.9	5	296	13.1	12.7 ± 0.8
		18 ♂	1.5	5.6	4.1	5	304	13.4	
		19 ♂	1.6	5.7	4.1	5	304	13.4	
		22 ♂	1.6	7.4	5.8	6	534	10.9	
Dried beef muscle	18	25 ♂	1.7	9.4	7.7	7	786	9.8	9.7 ± 0.5
		31 ♂	1.6	11.2	9.6	7	898	10.6	
		35 ♀	1.6	7.7	6.1	8	696	8.8	
Peanut flour	18	36 ♀	1.6	5.0	3.4	8	449	7.5	5.4 ± 1.2
		27 ♂	1.5	3.5	2.0	7	381	5.2	
		28 ♂	1.6	2.6	1.0	7	281	3.5	
Gluten	18	30 ♀	1.7	2.7	1.0	7	269	3.9	
		29 ♂	1.5	2.7	1.2	7	253	4.7[2]	
	40	37 ♂	1.6	6.0	4.4	8	950	4.6	4.4 ± 0.2
		32 ♂	1.6	6.7	5.1	8	1186	4.3	
Dried egg albumin	18	24 ♀	1.6	2.7	1.1	7	318	3.4	3.3
		26 ♂	1.6	2.7	1.1	7	339	3.2	
	40	33 ♂	1.6	2.1	0.5	8	888	0.6	1.2
		34 ♂	1.6	3.6	2.0	8	1156	1.7[3]	

[1] Omitted from average.

[2] Died in a convulsive seizure on 92nd day.

[3] Received heated egg albumin from the 45th day.

[4] Standard error.

to 58. This is similar to the effect observed by others in rats, in which supplementation with methionine above certain levels inhibited growth and protein utilization (Brown and Allison, '48).

Weast, Groody and Morgan ('48) reported that crude casein was definitely reduced in dog growth value when heated to 140°, due to loss of 30 to 40% in digestion. The heated washed casein in the present experiment did not produce the

TABLE 2

Protein indexes and absorption of egg yolk and 6 partially purified proteins

PROTEIN AND LEVEL IN THE DIET	APPARENT ABSORPTION	COMPARATIVE INDEX [1]	GRAM GAIN/GM N INTAKE	
			Observed	Calculated to 100% absorption
	%		*gm*	*gm*
Raw egg yolk, 12%	87 ± 2	100	12.7	14.6
Defatted whole egg, 12%	82 ± 3	75	9.6	11.7
Heated defatted egg, 12%	61 ± 4	63	8.1	13.3
Unheated casein, 18%	93 ± 1	74	9.4	10.1
Heated casein, 18%, plus 0.6% lysine	78 ± 1	70	8.9	11.4
Heated casein, 18%	76 ± 2	68	8.7	11.4
Unheated casein plus 1.0% methionine, 18%	91 ± 1	58	7.3	8.0
Dried beef muscle, 18%	89 ± 2	76	9.7	10.9
Peanut flour, 18%	88 ± 2	42	5.4	6.1
Gluten, 18%	94 ± 2	34	4.3	4.6
Gluten, 40%	96 ± 2	35	4.5	4.7
Dried egg, 18%	41 ± 6	26	3.3	8.0
Albumin, 40%	49 ± 5	9	1.2	2.4

[1] Egg yolk = 100.

same results, perhaps because the process of purification so altered the protein that the heating produced little additional change. The growth value of crude casein has been found (Greaves, Morgan and Loveen, '38) to be reduced by water washing alone. The decrease in biological value of the washed casein following dry heating was, however, less than that seen in similarly heated unwashed casein. This may be due to the complete removal of lactose by the washing process,

since lactose has been found in this laboratory and elsewhere (Mader et al., '49) to decrease the growth value of both casein and lactalbumin when heat is applied to the mixture. A similar effect by glucose (Stevens and McGinnis, '47) and sucrose (Evans and Butts, '48) has been reported.

Gluten fed at a 40% level produced the same growth as when fed at the 18% level. One of the dogs on the 18% gluten diet was seen to have 4 running fits. The last of these was of epileptiform type and was the cause of death on the 92nd day. Another dog, a female, was observed in only one running fit and was in better condition throughout than was the male. The dogs on the 40% gluten diet were observed in 7 and two running fits, respectively. The gluten was known to have been made from agenized flour.

Increasing the level of dried egg albumin from 18% to 40% produced a much decreased index value, from 26 on the former level to 9 on the latter. This was probably due to the increased intake of an antitryptic substance known to be present in undenatured egg white (Lineweaver and Murray, '47). The diet of one of the dogs was heated to 73° beginning on the 45th day, in the hope of decreasing the antitryptic effect. The dog gained weight at a somewhat faster rate than did the other on this diet, whose growth it had paralleled up to this point (fig. 1). Nevertheless, quantitative examination of the egg albumin for antitryptic activity, presumably due to ovomucoid, indicated relatively little change due to heating at 73°. All 4 of the dogs on the albumin diets had very poor hair growth and superficial skin ulcerations (Morgan et al., '51). The growth of two typical litters on some of these diets is shown in figure 1.

Apparent nitrogen absorption

Comparisons of the apparent absorption values shown in table 2 must be made chiefly among the proteins fed at the same level, since true digestibility is affected by the total volume of food as well as by the quantity and character of the protein. However, the correction necessary between the

12 and 18% levels is probably not large. Gluten, peanut flour, beef muscle, raw egg yolk, casein plus methionine and casein all had comparable apparent absorption values, the range being from 87% for the raw egg yolk to 96% for the gluten

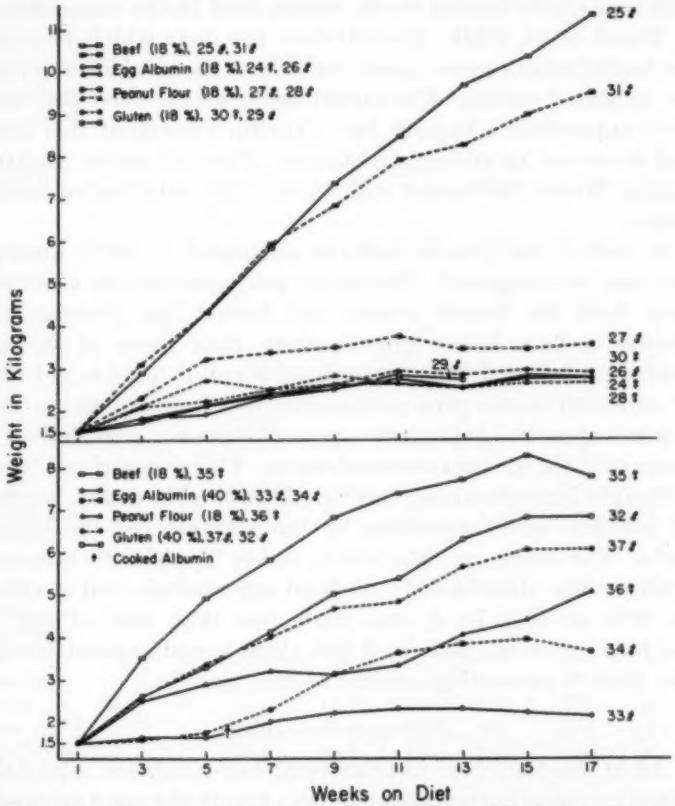

Fig. 1 Growth of two typical litters of purebred cocker spaniels on various food proteins.

(table 2). The value for egg powder was only 82%, an observation not in accord with the reports in the literature on rodent studies (Mitchell and Block, '46). The latter values were, however, for true rather than apparent digestibility.

Heat treatment of casein and egg decreased the apparent absorbability considerably, 10 to 20% for the casein and 20 to 25% for the defatted whole egg powder. The absorption of this heated casein was significantly better than was found with a similarly heated crude casein, used in the experiments of Weast et al. ('48). Nevertheless the dogs which received the heated casein grew almost as well as those which received the unheated casein. The amino acids which were absorbed were apparently adequate for optimum growth at this level and were not in critical imbalance. This is similar to findings by Mader, Schroeder and Smith ('49) with heated lactalbumin.

In table 2 the protein indexes calculated to 100% absorption may be compared. The amino acid assortments absorbed from both the heated casein and heated egg proteins appeared to have better growth value than those of the unheated proteins, if this comparison is valid. This is not true of unheated casein plus methionine, in which the amino acid mixture absorbed apparently supported poorer growth than in the case of the unsupplemented casein. This comparison, based on weight increase alone, may be misleading, however, because of possible water retention by the animals fed the heated diets. The serum protein levels (table 3) point to this possibility. The absorbability of dried egg albumin, fed at either an 18% or 40% level, was much less than that of any of the other proteins used, and this alone would account for the low growth-promoting quality of this protein.

Nitrogen retentions

All of the dogs were in positive nitrogen balance. The daily nitrogen retention per kilogram was nearly the same on casein, casein plus methionine and raw egg yolk, and only slightly less on the heated casein with or without added lysine and on defatted whole egg (fig. 2). These average values were found to present statistically significant differences. The heated egg, peanut flour, and egg albumin allowed significantly lower retentions than any of the others, the losses being

in the feces on the heated egg, egg albumin, and the heated caseins, and in the urine on peanut meal. The few values obtained for beef and gluten indicated lower nitrogen retention for the former and higher for the latter than the growth rates would indicate.

TABLE 3

Blood composition of young dogs after three to 4 months on the diets

DIETARY PROTEIN	NO. OF DOGS	NO. OF ANALYSES	HEMATOCRIT	HEMOGLOBIN	RED BLOOD CELLS	TOTAL SERUM PROTEIN	SERUM ALBUMIN	ALBUMIN TO GLOBULIN RATIO
			% cells	*gm %*	*millions per mm³*	*gm %*	*gm %*	
Unheated casein	3	12	43.5	14.2	6.9	5.46	4.08	2.9
Unheated casein plus methionine	2	8	43.0	14.1	6.9	5.44	3.93	2.6
Heated casein plus lysine	3	12	41.5	13.3	6.9	5.07	3.74	2.7
Heated casein	3	12	39.7	13.0	6.1	4.93	3.70	3.0
Raw egg yolk	5	11	41.6	13.6	6.4	5.47	3.56	1.8
Unheated dried whole egg	4	12	39.2	13.1	6.5	5.61	3.87	2.2
Heated dried whole egg	4	11	34.9	12.3	5.7	4.42	2.63	1.4
Dried beef muscle	2	4	41.3	13.1	6.9	5.37	3.88	2.6
Gluten	3	6	38.9	12.0	6.6	5.17	3.06	1.4
Peanut flour	3	6	30.6	9.6	4.8	5.26	2.83	1.1
Dried egg albumin	4	8	32.2	9.4	5.7	4.83	2.18	0.8

Blood composition

The dogs on unheated casein with or without methionine, whole egg powder, raw egg yolk, beef muscle, peanut flour and 40% gluten had approximately the same levels of serum

protein (table 3). An apparently high value for those fed the 40% albumin diet was undoubtedly due to hemoconcentration, as both these dogs had severe edema at the time of autopsy. Heat treatment of both casein and egg decreased the serum protein levels significantly. In figure 3 typical

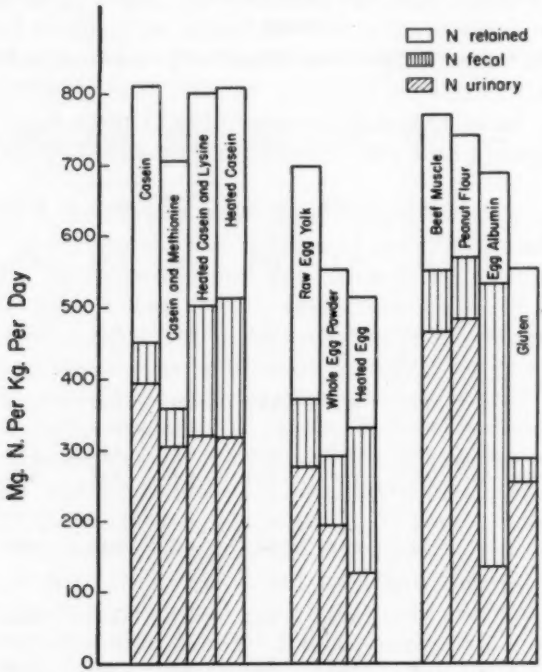

Fig. 2 Nitrogen exchange of young dogs on various food proteins, all fed at the 18% level except the egg diets, which were 12% protein. The third group, beef muscle, peanut flour, egg albumin and gluten, represents a small and possibly not statistically significant number of balances.

serum protein values for litter mates on unheated and heated casein and egg diets are shown.

The dietary protein appeared to govern the proportions of albumin and globulin in the serum. Thus all casein-fed dogs showed values of the albumin-to-globulin ratio close to 3.0,

compared with values near 2.0 in the animals fed the unheated egg diets. These figures refer to the levels of circulating proteins, not to the total amounts in circulation. Depressed albumin-to-globulin ratios were seen only in the dogs fed the heated egg, gluten, peanut flour and egg albumin. No striking differences in hemoglobin, packed cell volume, red blood cell counts or mean corpuscular volume were observed,

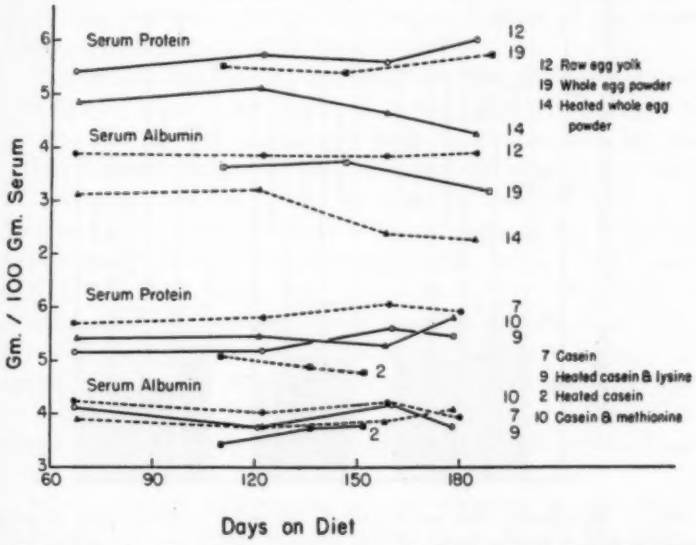

Fig. 3 Total serum protein and serum albumin levels of 7 typical young male dogs fed egg and casein diets.

although the dogs fed the heated proteins and the gluten, peanut meal and egg albumin diets had generally lower values in these respects (fig. 4).

The blood serum of the dogs fed raw egg yolk was analyzed for free and total cholesterol [4] in order to see whether feeding raw egg yolk at a 12% protein level seriously affected the metabolism of cholesterol. All values were within

[4] The cholesterol analyses were done by Marian E. Tolbert.

the normal range; 184 to 216 mg % in the dogs fed the defatted egg powder and 216 to 291 in those fed the raw egg yolk.

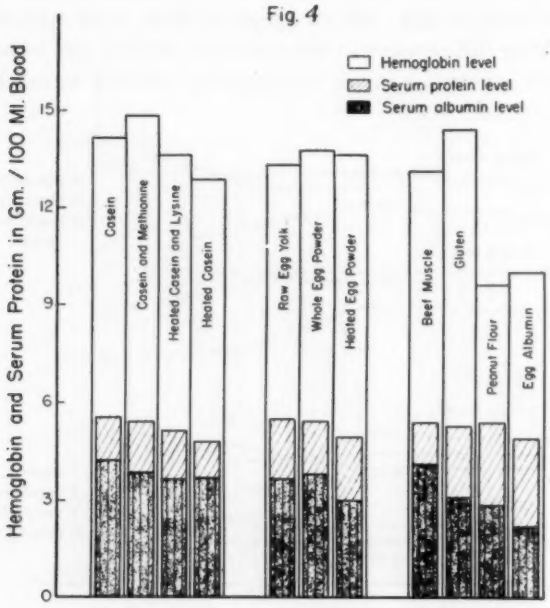

Fig. 4 Hemoglobin, total serum protein and serum albumin levels of young dogs fed various food proteins.

Condition of the tissues

Pale yellow soft livers were found in 4 of the 6 dogs fed heated casein, in one of the three dogs on egg powder, in both of those on heated egg powder and in one of those on raw egg yolk. All of the dogs on egg albumin and peanut meal had abnormally yellowish and soft or fibrous livers. The fat content of the fresh liver was found to be normal, however, except for one of the dogs on heated egg with 21% liver fat and two of the animals which received the

egg albumin. These had 9 and 24% fat in the fresh livers, as compared with 1.6 to 2.6% in the livers of the other 28 dogs examined.

The nitrogen content of liver, 2.23 to 2.98%, heart 2.46 to 2.74%, and kidney 2.64 to 2.82%, was of comparable range in all the dogs. The muscle of all the dogs was normal as to nitrogen, 2.85 to 3.07% fresh weight, and creatine content, 238 to 330 mg %. All of the dogs on the egg albumin diets had the lowest range of nitrogen content of the tissues, with relatively high moisture contents.

The female fed the raw egg yolk diet had a hardened aorta, and fibroid tissue was observed in the kidney of another dog on this diet as well as in both dogs fed the heated egg diet.

The average weight of the testes per kilogram body weight of the 5 dogs fed the heated diets at 199 and 239 days of age was markedly less than that of the 7 dogs of the same ages fed the unheated casein and egg diets, the averages being 0.50 ± 0.12 and 1.25 ± 0.17 gm, respectively. No such difference was seen among the 8 animals sacrificed at 154 to 170 days of age, the average weight being 0.36 ± 0.02. The sexual maturation of male dogs of this breed has been noted to occur at about 200 days of age. This development was apparently largely inhibited in the animals given the heated casein and egg diets.

No significant differences in the weights of the ovaries in proportion to body weight occurred on any of the diets in either of the two age ranges used, the average being 0.063 ± 0.007 gm per kilogram body weight in the 11 animals studied.

The biological value of these protein preparations has been reported upon by Allison, Anderson and White ('49), who used normal and protein-deficient adult dogs and expressed the results in terms of the "nitrogen balance index." The indexes of egg white, casein, and gluten were 1.12, 0.88, 0.74 and 0.44, respectively, for normal adult dogs and 1.12, 1.06, 0.84 and 0.70 for dogs depleted of protein. The raw egg white was much less digestible for the depleted than the normal animals and did not replete the dogs adequately. Apparently

the capacity of the depleted dogs' gastric secretion was decreased below the level required for the denaturation of the antitryptic substance in egg white. The comparative values obtained in the present experiment by the growth of young dogs, with the exception of those for the egg white, are in good agreement with those reported by Allison, Anderson and White ('49).

Chow, Alper and DeBiase ('48) have also reported on the regeneration of plasma proteins in protein-depleted adult dogs on oral administration of 4 of these protein preparations — whole egg, egg white, casein and gluten. The comparative values obtained in this way were similar to those obtained in the same laboratory by nitrogen balance, growth promotion in mice, replacement of body tissues and liver regeneration, except for egg white, which was superior to the other three in all but liver regeneration and production of plasma protein.

These plasma protein regeneration experiments, as well as the nitrogen balance studies of Allison, Anderson and White ('49), were done with adult dogs, as compared with our observations on young dogs, 6 weeks up to 8 months of age. Obviously the young dogs resemble the depleted animals reported by the latter authors in their inability to digest and absorb the raw dried egg albumin. The low dietary level of albumin in the balance studies also favored its utilization. The other 5 proteins were utilized for growth by dogs in much the same order reported for rats by Ruegamer, Poling and Lockhart ('50) and Mitchell and Beadles ('50) and for maintenance of nitrogen balance by human subjects by Hawley et al. ('48).

SUMMARY

Six partially purified proteins and the protein of fresh raw egg yolk were compared for their growth-promoting value for purebred young cocker spaniel dogs. Two of the protein sources, casein and dried defatted whole egg, were fed both unheated and heated at 140° for 15 minutes before being incorporated in the diets. Lysine (L + monohydrochloride) and

DL-methionine were added at 0.6% and 1.0% levels, respectively, to additional diets containing heated and unheated casein. All the egg diets were fed at a 12% level, all the casein diets, beef, peanut flour, gluten and egg albumin at the 18% level, and the gluten and egg albumin also at the 40% level.

On the basis of gain per gram nitrogen intake in 120 days from beginning weights of 1.5 to 2.0 kg, the egg yolk produced the best growth. If the egg yolk protein index is taken as 100, the other proteins had indexes as follows at 18 or 12% levels: beef 76, defatted dried whole egg 75, casein 74, peanut flour 42, gluten 34, dried egg albumin 26. At the 40% level the gluten had the value 35 and the egg albumin 9.

The heat treatment had little depressing effect on the casein, index 68, and the added lysine little reparative action, index 70. This may be due to the removal of lactose in the purifying process and the consequent prevention of a browning effect by heat. The value of the egg protein was decreased by heat treatment to 63. The addition of 1.0% methionine to the unheated casein led to depression of the protein index to 58.

The average absorption of all heated proteins was lowered 15 to 21% below that of the corresponding unheated proteins, but the reduction in growth value was relatively small. The levels at which the egg and casein diets were fed had been found to be the minima on which normal growth could be obtained. The amino acid assortments absorbed from the heated diets were apparently at least as favorable to growth as were those of the unheated proteins.

The egg albumin contained an active antitryptic substance which greatly reduced its absorbability and allowed very little growth.

The gluten diets were well absorbed, as were the peanut flour and beef. The loss of nitrogen in the gluten and peanut flour-fed dogs was in the urine.

The serum protein levels of the dogs on the heated diets and those on peanut, gluten and egg albumin were depressed, as were the albumin-to-globulin ratios on the heated egg,

peanut, gluten and egg albumin. The hemoglobin levels were also low on these 4 diets.

The tissues of all the dogs fed egg albumin were edematous and their livers were moderately fatty. The testes of the dogs at 200 days of age and over weighed significantly less per kilogram body weight in the animals on the heated diets. All the gluten-fed dogs had running fits and one died during a convulsive seizure.

These observations are in contrast with the superior performance of rats and adult dogs fed egg albumin at low levels in other experiments.

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EFFECT OF PYRIDOXINE DEFICIENCY ON REPRODUCTION IN THE RAT [1]

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ONE FIGURE

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Nelson and Evans ('48) have shown that the addition of the pyridoxine antagonist, desoxypyridoxine, to a pyridoxine (B_6)-deficient diet resulted in a high incidence of resorptions when normal adult rats were placed on the diet only 10 to 20 days prior to breeding. This was the more surprising since preliminary studies had shown that mere omission of the vitamin from a purified diet had only slightly adverse effects on reproduction. The present communication reports (1) the reproductive performance of rats maintained on three different B_6-deficient diets; (2) the effect of food restriction on reproduction in the presence of the antagonist; and (3) the effect of the vitamin deficiency on the young produced under these conditions.

EXPERIMENTAL

Normal female rats (Long-Evans strain) approximately three months of age were bred with normal males and placed on the B_6-deficient diets the day of mating. Additional groups

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of animals of the same age were placed on the diets for two to 4 weeks (sometimes for a longer period) and then bred with normal males. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign of implantation; the rats were weighed at regular intervals. All rats were autopsied a few hours after parturition had taken place except that in some instances when weight loss or other signs indicated that resorption was in process the autopsies were performed as early as the 20th day of gestation. At autopsy, the uterus was carefully examined for the presence of implantation sites, whether normal or in the process of resorption.

Three different B_6-deficient diets were used in this study. Pyridoxine was omitted from the vitamin mixture in all three diets and in one of the three groups no further alteration was made; succinylsulfathiazole (SST) was added to one diet to repress intestinal synthesis, and desoxypyridoxine, a potent B_6 inhibitor, to another diet. The basal B_6-deficient diet was the same as that used previously (Nelson and Evans, '48). It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil,[2] and 4% salts.[3] Crystalline vitamins per kilogram of diet were: 300 µg *d*-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5.5 mg pteroylglutamic acid, 10 mg riboflavin, 10 mg *p*-aminobenzoic acid, 20 mg nicotinic acid, 50 mg *d*-calcium pantothenate, 400 mg inositol and 1.0 gm choline chloride. Either 0.5 mg% desoxypyridoxine was added to this diet or 1% succinylsulfathiazole (SST) was included with a corresponding change in sucrose content. Control rats received the diet containing the antagonist supplemented with pyridoxine HCl at two levels, 0.5 mg% or 5.0 mg%. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg synthetic alpha-tocopherol and 650 mg corn oil.[4]

[2] Crisco or Primex.

[3] Salts 4 of Hegsted et al. ('41).

[4] Mazola.

RESULTS

Table 1 summarizes the reproductive performance of rats maintained on the three different B_6-deficient diets for varying periods of time. Omission of the vitamin from the diet when the deficiency was instituted on the day of breeding had only slightly adverse effects on reproductive performance. These effects included an increased percentage of young born dead, i.e., 6% instead of the normal 1 to 2%, and the failure of the mother to make the normal weight gain during gestation, i.e., 72 gm instead of the normal 100 gm or more. The occurrence of resorption in this group was presumably due to the unusual sensitivity of one rat to the vitamin deficiency, as no other resorptions occurred on this diet even after extended periods of deficiency prior to breeding. Neither did any resorptions occur in the group receiving the deficient diet containing SST, or in a preliminary group receiving the deficient diet from which biotin and pteroylglutamic acid had also been omitted.

A deficiency period of three weeks prior to breeding did not accentuate the deficiency sufficiently to affect reproductive performance, and a rebreeding of the original group of animals after approximately two months (54 days) on the deficient diet resulted only in a further decrease of the weight gain during gestation (i.e., 48 gm in comparison with 62 to 72 gm).

The addition of SST to repress intestinal synthesis did not accentuate the vitamin deficiency or result in any greater reproductive impairment. The maternal weight gain during gestation was decreased by two months of prior deficiency as in the previous groups on the basal deficient diet.

Effect of the antagonist

The addition of desoxypyridoxine to the deficient diet on the day of breeding resulted in as acute a vitamin deficiency as that produced by two months of deficiency prior to breeding on the other deficient diets. Furthermore, a short period of deficiency prior to breeding resulted in the same high incidence

TABLE 1

Reproduction of rats maintained on pyridoxine-deficient diets

DEFICIENCY PRIOR TO BREEDING	RATS BRED	BODY WT. CHANGE		RBC [2]	FAILED IMPLANTATION	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	YOUNG BORN DEAD	AVE. YOUNG PER LIT.	AVE. WT. YOUNG
		Wt. at mating	During gestation [1]							
days	no.	*gm*	*gm*	*day*	%	%	%	%	no.	*gm*
				B_6-deficient diet						
0	10	207	+ 67	13.0	0	10	90	6	9.9	5.1
22	12	234	+ 62	13.7	0	0	100	1	7.8	5.7
54 [4]	8	245	+ 48	14.4	0	0	100	7	7.8	5.5
				B_6-deficient diet + SST						
0	10	209	+ 82	13.5	0	0	100	1	9.0	5.4
58 [3]	6	238	+ 45	14.4	0	0	100	3	6.3	5.6
				B_6-deficient diet + desoxypyridoxine						
0	10	214	+ 39	13.9	0	0	100	3	6.8	5.4
0	11	212	+ 38	13.2	0	9	81	3	7.6	4.7
12	11	205	+ 12	12.6	0	36	64	0	5.7	5.2
16	12	212	[5]	11.4	0	75	25	21	6.3	4.6
18	13	234	+ 9	13.2	0	46	54	0	6.6	4.8
20	8	214	+ 2	11.3	0	63	37	6	5.3	3.8
22	7	222	— 14	11.8	29	100	0			
24	10	216	— 14	11.8	0	90	10	0	2.0	4.0
				B_6 control diet + desoxypyridoxine						
14 [4]	10	208	+ 97	13.1	0	0	100	0	9.3	5.6
20 [4]	13	203	+ 111	12.8	0	0	100	0	8.9	5.8

[1] Data determined during the 20 days following mating.
[2] Day when vaginal smear first contained erythrocytes (RBC).
[3] This is the second breeding for these rats.
[4] These groups were shifted to the B_6 control diet the day of breeding and averaged 14 to 20 days of deficiency prior to breeding.
[5] The majority of the rats in this group were autopsied prior to the 20th day of gestation.

of resorptions as was previously reported (Nelson and Evans, '48). The percentage of resorptions increased directly with extension of the prior deficiency period, although there was considerable variability among groups of rats studied at different times. In the first large series of animals studied, 36% of the animals resorbed after 12 days of prior deficiency, 75% after 16 days, and 100% after 22 days. In the second large series of animals, 46% resorbed after 18 days of prior deficiency, 63% after 20 days, and 90% after 24 days. The second series of animals were slightly heavier in body weight and may be presumed to have had larger stores of vitamin B_6. Succeeding groups of animals have shown the same picture of variability with the shorter periods of deficiency but 90 to 100% resorptions consistently after three weeks of deficiency prior to breeding. It should be mentioned that longer periods of deficiency have not been used because of the extremely early loss of the estrous cycle in rats maintained on the deficient diet containing the antagonist, i.e., in 15 to 25 days. When breeding was begun after 18 days of deficiency, usually only 40 to 50% of the animals could be successfully bred.

The tendency toward a decreased number of young per litter may be noted in all deficient groups receiving the antagonist, as well as in the other groups with prior deficiency. These deficient groups had a combined average of 7.0 ± 0.3 young per litter, based on 65 litters. In contrast, the control groups together with the remaining deficient groups without prior deficiency and without the antagonist averaged 9.2 ± 0.3 young per litter, based on 42 litters. The tendency toward a decreased weight of the young at birth, i.e., less than 5 gm, may also be noted in many of the deficient groups receiving the antagonist. One group of rats receiving the antagonist, with 22 days of prior deficiency, showed an abnormal failure of implantation (29%). This occurred occasionally but not consistently in succeeding groups. A marked decrease in maternal weight gain, and even weight loss, during gestation occurred in the groups receiving the antagonist.

The early appearance of erythrocytes in the vaginal smear, even as early as the 8th day after breeding, was characteristic for resorbing animals receiving the antagonist. This is shown in table 1 by the average appearance of vaginal erythrocytes before the 12th day in most of the deficient groups with prior deficiency. Figure 1 presents the vaginal smears for typical deficient and control animals and illustrates the variability shown by deficient animals. Unpublished data for additional control and stock animals indicates that the macrosopic appearance of vaginal erythrocytes in the Long-Evans strain before the 12th day invariably means the presence of a resorbing site. Similarly of interest was the occasional appearance of cornified vaginal smears, which usually followed an early appearance of vaginal erythrocytes. These cornified smears presumably indicated the end of gestation, i.e., degeneration of the placenta. The rapidity of the resorptive process was another characteristic of deficient rats receiving the antagonist. This process was carried out so rapidly that when the autopsy of the animals was delayed until the end of gestation, i.e., the 20th to 24th days, no trace of implantation sites could be observed in many cases (see fig. 1). Nevertheless, if such animals were autopsied at the first appearance of vaginal erythrocytes, implantation sites were always observed.

Deficient animals receiving the antagonist were further characterized by a loss of the estrous cycle in 15 to 25 days, as previously mentioned, the appearance of acrodynia of the paws at the same time, and an extremely low mortality, less than 5% during 60 days of deficiency. Generally, body weight was maintained for 30 to 40 days, after which a gradual loss in weight occurred.

Effect of food restriction on reproduction in the presence of the antagonist

To eliminate the factor of undernutrition as a cause of this marked reproductive failure in the presence of the antagonist,

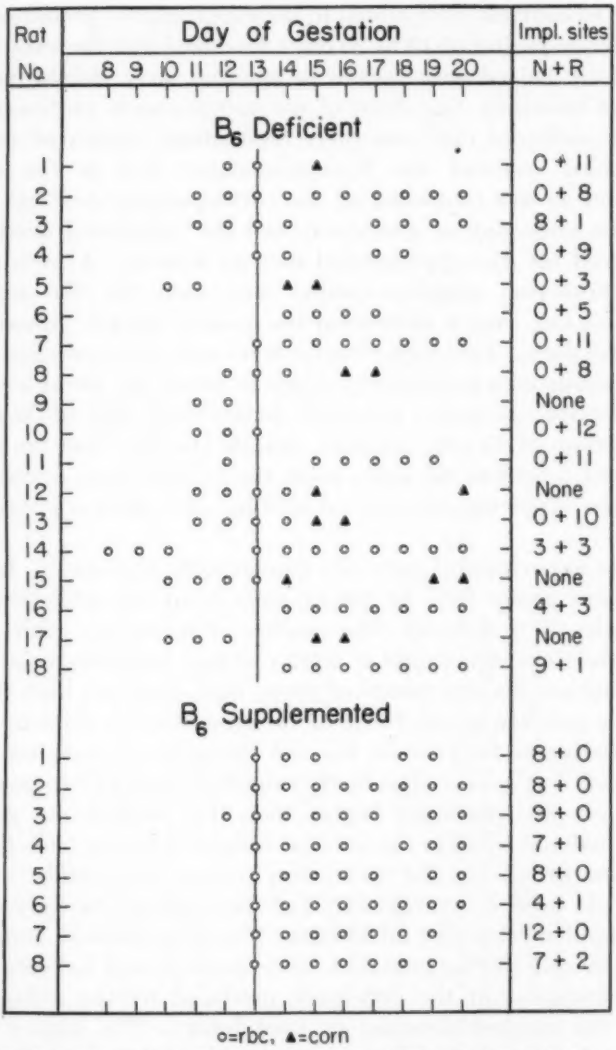

Fig. 1 Appearance of erythrocytes (rbc) or cornified cells (corn) in the vaginal smear during gestation for B_6-deficient and supplemented rats. Both diets contained the antagonist, desoxypyridoxine. The number of inmplantation sites (Impl. sites) which were normal (N) or resorbing (R) for each rat is also given.

pair-fed controls were added to the experimental procedure. A deficiency period of 19 to 20 days was used and the rats were carefully paired with regard to age and body weight on the day of breeding. One-third of the animals were continued on the B_6-deficient diet and their food intake measured daily; one-third received the B_6-supplemented diet in the same amount as was consumed by the corresponding deficient rats on the same day of gestation; and the remaining one-third received the B_6-supplemented diet ad libitum. A high level of pyridoxine supplementation was used for the control groups, i.e., 5 mg% or tenfold the amount usually present in control diets. This high vitamin level was made necessary by the results of a preliminary study in which the usual level of pyridoxine (0.5 mg%) was used. In this study 23% of the pair-fed group of 13 rats resorbed despite the fact that the same vitamin level was adequate when the animals were allowed to eat the supplemented diet ad libitum (see control groups in table 1).

The experimental data are summarized in table 2. In the deficient group 63% of the animals resorbed, while the remainder, 37%, littered. The number of young per litter (5.3) and their average weight at birth (3.8 gm) were below normal; in addition, the percentage of young born dead was high (6%). In the pair-fed group 100% of the rats littered, showing that restriction of food intake was not the cause of resorptions in the deficient group. The birth weight of young from pair-fed mothers was markedly higher than that of deficient young, although not equal to the average weight of young from the ad libitum group. In the restriction regime food intake varied from 44 to 68% (average 56%) of the intake of rats given the B_6-supplemented diet ad libitum. The differences in maternal weight gain during gestation were marked, and indicate both the acuteness of the deficiency produced by the antagonist and the marked decrease in food intake. The higher than normal percentage of young born dead in both the pair-fed and ad libitum control groups suggests that the already high level of vitamin supplementation should have been further increased.

TABLE 2

Effect of food restriction on reproduction in the presence of desoxypyridoxine [1]

EXPTL. GROUP	RATS BRED	BODY WT. AT MATING	WT. CHANGE DURING GESTATION [2]	AVE. DAILY FOOD INTAKE [2]	VAGINAL RBC [4]	FAILED IMPLANTATIONS	RESORPTION OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	YOUNG BORN DEAD	AVE. YOUNG PER LITTER	AVE. WT. OF YOUNG
	no.	*gm*	*gm*	*gm*	*day*	*%*	*%*	*%*	*%*	*no.*	*gm*
B_6-defic.	8	214	+ 2.1 ± 2.8 [5]	9.5 ± 0.4	11.3 ± 0.7	0	63	37	6	5.3 ± 1.9	3.8
B_6-suppl. pair-fed controls	8	212	+ 39.5 ± 5.3	9.5 ± 0.4	12.3 ± 0.3	0	0	100	3	7.9 ± 0.8	5.6
B_6-suppl. ad libitum controls	8	212	+ 113.3 ± 10.1	17.3 ± 0.7	12.9 ± 0.1	0	0	100	5	7.1 ± 0.9	6.2

[1] All groups averaged 19 to 20 days of B_6 deficiency prior to breeding.
[2] Data determined during the 20 days following mating.
[3] Data determined during 21 to 22 days following mating.
[4] Day when vaginal smear first contained erythrocytes (RBC).
[5] Standard deviation (error) of the mean.

TABLE 3

Growth and survival of young from mothers maintained on pyridozinc-deficient diets

EXPERIMENTAL DIET	NO. OF YOUNG	AVE. WT. DAY 1	DAY 10		DAY 15		DAY 21	
			Ave. wt.	Survival	Ave. wt.	Survival	Ave. wt.	Survival
		gm	*gm*	%	*gm*	%	*gm*	%
			Control diet instituted at birth					
Controls	342	6.2	22	97	32	97	49	97
			Deficient diet instituted at birth					
Deficient [1]	60	6.3	21	100	24	85	25	28
Deficient	72	6.5	22	99	22	94	21	10
Deficient + SST	60	5.9	21	98	22	93	24	17
			Deficient diet instituted first day of gestation					
Deficient [1]	60	5.5	14	77	17	13	..	0
Deficient	54	5.4	17	81	13	39	..	0
Deficient + SST	60	5.6	15	97	15	20	..	0
Deficient + desoxypyridoxine	53	5.5	12	60	13	36	16	6

[1] Synthetic biotin and pteroylglutamic acid were omitted from the vitamin mixture used for this one diet.

Growth and survival of suckling young

The growth and survival of the suckling young born under these experimental conditions are shown in table 3, together with the corresponding data for young in which the deficiency was instituted at birth. The figures for young whose mothers were maintained on a B_6-supplemented purified diet during the lactation period are included for comparison.

When the deficiency was instituted at birth, growth was normal up to the 10th day of lactation and then practically ceased. Survival of the young was normal up to the 15th day, which is apparently the critical period with this procedure. In agreement with Daniels et al. ('42), epileptiform convulsions were observed in a high percentage of these young between the 15th and 18th days.

When the deficiency was instituted on the first day of gestation, growth of the young on all 4 deficient diets was markedly retarded by the 10th day and thenceforth no further growth ensued. Survival of the young was already slightly below normal by the 10th day and the majority died before the 15th day. The critical period under these experimental conditions was between the 10th and 15th days of lactation. The incidence of convulsions observed in these young was lower than in the young deficient only from birth. It should be noted that the addition of desoxypyridoxine during gestation and lactation did not accentuate the already acute deficiency observed in the young during the lactation period. This is in contrast to the markedly deleterious effect of the antagonist on the deficiency during gestation.

DISCUSSION

The data presented in this study show that pyridoxine is necessary for normal reproductive performance in the rat, particularly when its deficiency is accentuated by the presence of its antagonist, desoxypyridoxine. It is probable that the deficiency could be accentuated by other means, e.g., the use of younger rats or longer periods of deficiency prior to breeding.

The effect of this deficiency in voluntarily restricting the food intake (56%) observed in this study was greater than that found for the vitamin deficiencies previously studied; i.e., 69% for pantothenic acid deficiency (Nelson and Evans, '46), and 76% for pteroylglutamic acid deficiency in the presence of an antagonist (Nelson and Evans, '49). The lack of correlation between food intake and reproductive performance which has been pointed out for previous vitamin deficiencies was likewise shown in this study. In the paired-feeding experiment, the average food intake for three deficient rats that littered was 8.8 gm, in contrast to the average food intake of 10.0 gm for 5 deficient rats that resorbed.

The early appearance of vaginal erythrocytes and the rapidity of the resorptive process observed in this study distinguish this vitamin deficiency from the other deficiencies studied. The only dietary condition reported in which the process of resorption is as rapid is the acute folic acid deficiency produced by aminopterin injections (Thiersch and Phillips, '50). A similar early appearance of vaginal erythrocytes has been noted for other dietary deficiencies, e.g., protein deficiency (Guilbert and Goss, '32) and vitamin A deficiency (Mason, '35). The possibility that these or other dietary deficiencies were present in the pyridoxine-deficient rats of this study is eliminated by the normality of reproductive performance in the pair-fed controls. However, the possibility of a hormonal deficiency is suggested by the work of Pencharz and Long ('33), who found that hypophysectomy before the 10th day of gestation resulted in early vaginal hemorrhage and resorption of the fetuses. This possiblity is further indicated by the rapidity of the resorptive process in the bred rats, together with the early loss of the estrous cycle in unbred rats before any marked loss in body weight occurred.

SUMMARY

Reproduction has been studied in adult rats placed on different pyridoxine-deficient diets for varying periods of time. Only slightly adverse effects on reproduction were obtained by the

simple omission of the vitamin, even with two months of deficiency prior to breeding. The addition of succinylsulfathiazole to repress intestinal synthesis did not result in any greater reproductive impairment. In confirmation of previous studies, the addition of the antagonist, desoxypyridoxine, to the deficient diet resulted in a high incidence of resorptions with only 10 to 20 days of deficiency prior to breeding. The early appearance of erythrocytes in the vaginal smears and the extreme rapidity of the resorptive process distinguished this vitamin deficiency from others previously studied and indicated the possibility of hormonal deficiencies in these animals.

Pair-fed animals supplemented with high levels of pyridoxine demonstrated that food restriction, although marked, was not the cause of the resorptions.

The growth of suckling young born from mothers with a pyridoxine deficiency was markedly retarded early in the lactation period, and survival to weaning rarely occurred. Neither succinylsulfathiazole nor desoxypyridoxine accentuated the deficiency in such young during the lactation period. Epileptiform convulsions were observed in some of these young but occurred to a greater extent in young suckled by mothers from whose diet the vitamin was withdrawn on the day of birth.

ACKNOWLEDGMENTS

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THE EFFECT OF VITAMIN B_{12} ON THE RESPONSE OF CHICKS TO BETAINE AND CHOLINE [1]

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The recent trend toward the use of poultry feed ingredients rich in productive energy has stimulated a renewed interest in compounds which furnish labile methyl groups. Mishler, Carrick and Hauge ('49) showed that either betaine, choline or methionine effectively supplemented a practical chick ration composed principally of corn and soybean meal. They concluded, however, that 0.3 or 0.4% betaine in the diet promoted a faster rate of growth than supplements of either choline or methionine. Gillis and Norris ('49a, b) also demonstrated the supplementary effect of betaine or choline in rations similar to those used by Mishler et al.

The work by Gillis and Norris showed, furthermore, the important influence of vitamin B_{12} on the requirement for methylating compounds, and led to the suggestion that vitamin B_{12} is concerned in transmethylation. In an earlier paper, Patton et al. ('46) had reported that adding fish meal to an all-vegetable ration apparently lowered the requirement of the chick for methionine. Schaefer et al. ('49) have also presented evidence that the choline requirement of chicks receiving a diet containing a high percentage of fat is decreased by feeding vitamin B_{12}.

[1] We are indebted to the International Minerals and Chemical Corporation, Chicago, Illinois, for generous support of this project.

[2] Research Associate.

Dubnoff ('49a, b) and Muntz ('50) have presented evidence that choline must be oxidized to betaine before it can function as a methyl donor. In an exploratory experiment with vitamin B_{12}-deficient chicks, it was observed that betaine was a better supplement for a simple ration than was choline. In view of this observation and also the influence of vitamin B_{12} on the labile methyl requirement, it seemed possible that vitamin B_{12} is important in the enzymatic conversion of choline to betaine, or at least in some reaction necessary for the transfer of methyl groups from choline to other compounds. If vitamin B_{12} is concerned in choline utilization in a manner which does not hold true for betaine, differences in the efficiency of these compounds should be apparent in animals having suboptimum body stores of vitamin B_{12}.

In this paper the results of several experiments dealing with this problem will be presented.

EXPERIMENTAL

In order to determine the effect of vitamin B_{12} depletion on the utilization of betaine and choline by chicks, these compounds were added singly to a chick diet previously found to be improved by these supplements. The vitamin B_{12} reserves of the chicks used in this series of experiments varied from those encountered in good hatchery management to those of chicks made very deficient in this factor. The depletion was accomplished by confining the hens producing hatching eggs to wire-floored pens and feeding them a ration containing no animal protein. In some cases a more rigid depletion was attempted by feeding the chicks from such hens an all-vegetable ration for a pre-experimental period of three or 4 weeks. The indications were, however, that such a procedure was not very effective in producing further depletion of vitamin B_{12}. The chicks referred to as "normal" in this report were obtained from a commercial hatchery using a good breeder ration known to contain animal protein.

The basal chick diet used in all of these experiments had the following percentage composition: yellow corn meal 46,

crushed wheat 15, pulverized oats 5, soybean oil meal 30, fish liver oil 0.5, salt 0.5, dicalcium phosphate 2.0, and limestone 1.0. In addition the following supplements were added to each 100 lb. of the basal mixture: riboflavin 0.1 gm, niacin 1.0 gm, calcium pantothenate 0.5 gm, vitamin K (menadione) 0.1 gm, $MnSO_4 \cdot 4H_2O$ 10 gm.

Three experiments were carried out with normal male chicks; two with vitamin B_{12}-deficient chicks of mixed sexes, placed on the experimental diets at the time of hatching; and three were completed with vitamin B_{12}-deficient chicks of mixed sexes which received the basal diet for a pre-experimental period of three or 4 weeks. The number of deficient chicks used in an experiment was generally smaller than the number of normal chicks used because of the low rate of hatchability of vitamin B_{12}-deficient eggs and the subsequent high mortality of such chicks during the first week.

RESULTS

The results of these experiments are summarized in table 1. The results of all experiments with a given dietary supplement and with chicks of the same nutritional history with respect to vitamin B_{12} storage are combined.

The experiments with normal male chicks showed that the basal diet promoted a significantly faster rate of growth in chicks when supplemented with 0.2% of either betaine hydrochloride or choline chloride. The growth stimulation due to either of these compounds was essentially equal at the level fed. In one experiment with normal chicks, betaine was supplied in two different forms to separate lots. One lot received crystalline betaine hydrochloride, while another received an equivalent amount of betaine from the end liquor remaining after the removal of glutamic acid from concentrated Steffen's filtrate. The results with both sources of betaine were comparable.

Chicks which were hatched with subnormal body reserves of vitamin B_{12} responded to choline in a more variable manner. In two separate experiments in which the deficient chicks

were given the experimental diets immediately after hatching, a relatively small improvement in growth was obtained as a result of choline supplementation. In one experiment the chicks receiving choline weighed, on the average, 35 gm more than the basal lot, and in the second experiment only 16 gm more at 6 weeks of age. Chicks receiving betaine in the same experiments averaged, respectively, 102 and 45 gm

TABLE 1

Growth of normal and vitamin B_{12}-deficient chicks which received the basal diet unsupplemented or supplemented with betaine or choline

SUPPLEMENT	NORMAL CHICKS [1]	CHICKS [2] FROM B_{12}-DEFICIENT HENS	
		Wt. 6th week	Wt. gain, 4-wk. period [3]
	Ave. of 3 exps. gm	Ave. of 2 exps. gm	Ave. of 3 exps. gm
None	328 (81)[4]	275 (32)	143 (52)
0.2% choline-Cl	395 (75)	296 (35)	184 (53)
0.2% betaine-HCl	408 (107)	343 (31)	215 (53)
DIFFERENCES	%	%	%
Increase of choline groups over basal	20.4	7.6	28.7
Increase of betaine groups over basal	24.4	24.7	50.4
Increase of betaine groups over choline	3.3	15.9	16.8

[1] Males only.

[2] Mixed sexes.

[3] Chicks received basal diet for pre-experimental period of approximately three weeks.

[4] Figures in parentheses indicate number of chicks surviving experimental treatment.

heavier than the chicks in the basal lot. Under these conditions, therefore, betaine was a more effective supplement than choline.

One of the difficulties experienced in using small numbers of newly-hatched, vitamin B_{12}-depleted chicks is securing complete uniformity between experimental lots. This difficulty, however, was largely overcome in the case of chicks which

were placed on the basal diet for a pre-experimental feeding period of three to 4 weeks. During this preliminary period the weakest chicks were eliminated by natural mortality, and at the beginning of the experimental treatments the smallest and largest chicks were discarded. In this manner it was possible to secure very uniform lots of vitamin B_{12}-deficient chicks and largely to eliminate mortality during the experimental period.

Three experiments were completed with chicks treated and selected in this manner. The average gains in weight of all chicks in each treatment are shown in the last column of table 1. When the chicks were thus selected for rigid uniformity between lots and minimum variability within lots, the basal ration supplemented with either betaine or choline resulted in significantly greater gains in weight than were obtained with the unsupplemented ration. However, the average increase in weight due to betaine was nearly twice as great as the increase due to choline. There was more variability between experiments in the response of chicks to choline than there was in the response to betaine. In each case, however, the gain due to betaine was superior to that obtained with choline.

DISCUSSION

The results of these experiments afford further evidence that chick diets composed of mixed cereals and plant protein concentrates are improved by supplements of either betaine or choline. The comparative efficiency of betaine and choline in these experiments, however, was influenced by the state of nutrition of the chick with respect to vitamin B_{12}. Chicks from hens receiving a diet adequate in vitamin B_{12} responded to either of these compounds with an approximately equal increase in growth. Chicks with depleted reserves of vitamin B_{12}, however, responded to choline supplements with weight gains which were more variable and, on the average, significantly smaller than the gains due to betaine. In 5 experiments with depleted chicks betaine caused an average

increase in weight which was 16 to 17% greater than that for chicks receiving equivalent amounts of choline.

The reason for the greater efficiency of betaine for vitamin B_{12}-deficient chicks is not certain at present. It appears, however, that vitamin B_{12} plays a wider role in the utilization of choline than in the utilization of betaine. If vitamin B_{12} is necessary for the enzymatic conversion of choline to betaine, it would be logical to expect a decrease in choline oxidase activity in chick tissues deficient in vitamin B_{12}. Unpublished data of the authors, however, show that the livers of chicks deficient in vitamin B_{12} are a good source of choline oxidase. It appears, therefore, that vitamin B_{12} is concerned in the utilization of choline by the chick in some manner other than simple oxidation to betaine or the transfer of "labilized" betaine methyl. Dubnoff ('49a) has reported that chicks do not have choline oxidase in their tissues, while Dinning and associates ('50) recorded finding it in the bone marrow, liver and kidney tissue of the adult hen. The present authors' observations support those of Dinning et al. The effect of vitamin B_{12} on the activity of choline oxidase in the chick is being studied further.

There is further evidence that vitamin B_{12} has other functions in transmethylation aside from any possible role which it may play in the conversion of choline to betaine. Oginsky ('50) has reported that rat liver tissue forms more methionine from homocystine and betaine than from homocystine and choline. The process was less efficient in vitamin B_{12}-depleted liver tissue than in livers containing vitamin B_{12}, regardless of the source of methyl groups. Since the utilization of either betaine or choline for this process was improved by the administration of vitamin B_{12}, the conclusion was reached that this factor was concerned in the actual transfer of the methyl group.

The amount of choline naturally present in the ingredients of the basal diet used in these experiments was adequate for the prevention of perosis, as no cases of this syndrome were observed in any of the chicks. Under these conditions, there-

fore, the requirement for choline or betaine in the growth processes of the chick was greater than the requirement for the prevention of perosis. This appears to be in contrast to turkey poults, which have a higher requirement for the prevention of perosis than for a maximum rate of growth (Jukes, '40; Scott, '50).

SUMMARY

Under the experimental conditions reported above the addition of betaine or choline to a practical ration based on cereals and vegetable protein resulted in a significant improvement of the ration as measured by chick growth. In several experiments with White Leghorn chicks having normal body stores of vitamin B_{12}, the growth responses due to either betaine or choline supplements were approximately equal. Chicks having subnormal reserves of vitamin B_{12}, however, utilized betaine more effectively than choline. In 5 experiments with vitamin B_{12}-depleted chicks a significantly greater gain in weight resulted from adding betaine to the basal diet than from adding choline. The evidence suggests that at least one mechanism by which the chick utilizes choline is impaired by a deficiency of vitamin B_{12}, while betaine retains its effectiveness under the same conditions.

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EXPERIMENTAL DENTAL CARIES IN RATS

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In 5 experiments reported during the period 1930 to 1935, experimental diets containing from 40 to 80% glucose, sucrose, lactose or maltose failed to produce experimental dental caries in white rats (Jackson, '30; Lilly, '32; Lilly and Grace, '32; Day et al., '35). These diets were started at weaning age and were continued for from 63 to 365 days. The present author has previously reported (McClure, '45) some success and also some failure in producing a significant incidence of caries in rats fed a 70% sucrose diet starting at weaning age. More recently, Sognnaes ('47a, b, '48, '49) induced caries in rats given 67.0% sucrose diets, particularly when the diets were given to the females during gestation.

Cox, Dodds, Levin and Hodge ('48) concluded that sugars have little, if any, effect in starting rat caries, but that sugar does "provoke the rapid and profound advance of caries in the rat molar." Schlack et al. ('49) also found carious lesions in 10 rats given a 67% sucrose diet starting at weaning age. Shaw ('50) reported carious lesions among 72 rats given an adequate synthetic diet containing 67% sucrose; this diet was started at weaning age and continued for 14 weeks.

The hypothesis that refined sugars play a role in experimental caries is also supported by evidence that sugar in the diet of the Syrian hamster is conducive to the "initiation of lesions, as well as development" (Keyes, '46), and by results with cotton rats (Shaw, '50; Shaw et al., '44) in which carious teeth resulted from high sugar diets.

Much would be gained for dental research were it possible to produce dental caries consistently in an experimental animal, particularly in a laboratory animal as well standardized as is the white rat and by means of a synthetic diet of powdery consistency in place of a hard corn or rice particle diet. These latter diets cannot be standardized and raise a question as to the effect of the fractures induced by hard coarse particles (Hoppert et al., '32).

If current bacterio-chemical theories of caries etiolgy are correct (Miller, 1890; Leicester, '49), fermentable sugars should be caries-inducing. In addition to dietary factors, however, there is a major problem of inherent caries susceptibility in animals selected for caries studies. There is evidence from several sources that caries susceptibility is a litter (McClure and Arnold, '41; McClure, '45) and a strain characteristic (Hunt and Hoppert, '50; Hunt et al., '44; Schweigert et al., '45) and, more significantly, it has been shown by the notable work of Hunt, Hoppert and Erwin ('44) that inheritance factors are involved in the caries susceptibilty of rats. As a result of their extensive studies, these workers have developed "a uniform strain of rats known to be highly susceptible to dental caries when fed a specific diet."

In addition to the vital factors of inherent susceptibility and the composition of the diet, there is evidence, particularly in the studies by Sognnaes ('47b), that caries may not develop in mice and rats and will be limited in hamsters unless feeding of the experimental diet is begun during tooth development; i.e., during pregnancy and lactation. In other studies with hamsters (Mitchell and Shafer, '49) it is reported that "animals receiving a caries-producing diet initiated at various pre- and postnatal ages developed varying degrees of caries, most marked in those initiated at 8 to 30 days after birth."

The above studies thus call attention to problems related (a) to the role of the diet ingested during tooth development, and (b) to the possibility that young and newly erupted teeth may be particularly susceptible to an excessive amount of sugar in the diet. In the experiments reported below we have

been interested particularly in the latter of these two problems.

EXPERIMENTAL

Two diets, 560 and 561, were fed ad libitum. Diet 560 is entirely "synthetic" and adequate according to accepted standards. Diet 561 is not a "synthetic" diet; it was less palatable than diet 560 and did not support normal growth. Feeding was continued for 120 days, at the end of which time the rats were sacrificed and the teeth prepared for observation by autoclaving the head for 15 minutes to facilitate removal of the soft tissue. Caries was diagnosed grossly and scored according to the system of Cox, Dodds, Dixon and Matuschak ('39). The evaluation of caries was based on the number of carious teeth, the number of areas affected and the size of the cavities (as indicated by the caries score), and also on the number of rats within the group who had caries in one degree or another. Because of the factor of litter susceptibility it is essential that a number of litters be represented in each group. Tables 1 and 2 present the results of our studies and show the general plan of the experiments.

Experiments 1 and 2 (table 1) compare the effect of initiating the diet at birth vs. at weaning age; i.e., 21 to 25 days after birth. Among the 104 rats given diet 561 from birth, 69.2% exhibited dental caries. An average of 35% of the teeth of the affected rats were involved and they averaged a comparatively high caries score of 16.2. This is strikingly different from the results obtained in experiment 2, in which only 10% of the 91 rats exhibited dental caries and had a mean caries score of 5.8.

The caries susceptibility of the rats fed diet 560 in experiment 3 (table 2) was regarded as "high." The majority of these rats were the offspring of females sent to us by Dr. Hunt from the colony of Michigan State College and were from his caries susceptible strain (Hunt et al., '44). There were in addition 18 rats (4 litters) provided by Dr. C. A. Schlack from the rat colony of the Naval Medical Research Institute at

Bethesda, Md. Rats from this latter colony at that time gave a high incidence of corn meal-induced caries. In experiment 3 the experimental diet was given to all the females at the time the young were born. This group of rats gave the highest percentage of caries — i.e., 80.3% — the most carious teeth and the most severe caries, as indicated by the number of lesions and the score per affected animal.

TABLE 1

Dental caries in white rats receiving a 75% glucose diet starting at birth and at weaning

Experiment no.	1	2
Diet	561 [1]	561
Time of starting diet	at birth	weaning
Days on diet	120	120
Strain of rats	N.I.H.[2]	N.I.H.[2]
Caries susceptibility	undetermined	undetermined
Number of rats	104	91
Number of litters	19	16
Ave. number of rats per litter	5.5	5.7
Rats with caries	72	10
Rats with caries (%)	69.2	10.9
Carious teeth per carious rat	4.2	2.5
Carious teeth per carious rat (%)	35.0	20.8
Ave. number of carious areas per carious rat	6.5	3.1
Caries score per carious rat	16.2	5.8

[1] Diet 561: Cerelose 75.0%, whole milk powder 23.0%, salt 2.0%, Fe as ferric citrate 100 p.p.m., Cu as copper sulfate 10 p.p.m. (raw carrots weekly).

[2] National Institutes of Health.

The caries obtained in experiments 4 and 5 was less extensive, only 12% and 18%, respectively, and the rats that were affected had a less severe type of caries than the rats in experiment 3. The explanation of this difference appears to rest largely on the factor of inherent susceptibilty. The females which gave birth to the rats in experiment 5 (table 2) were continued on diet 560 through another pregnancy, which resulted in the 54 rats studied in experiment 6. These two experiments (5 and 6) thus serve to compare the effect of diet

TABLE 2

Dental caries in white rats receiving an adequate synthetic diet containing 72.6% glucose. A comparison of a caries-susceptible strain of rats (Michigan and Navy) vs. two strains of rats of unknown caries susceptibility (N.I.H.[1] and Sherman)

Experiment no.	3	4	5	6
Diet	560 [2]	560	560	560
Time of starting diet	at birth	at birth	at birth	prenatal
Days on diet	120	120	120	120
Strain of rats	Mich.-Navy	N.I.H.	Sherman	Sherman
Caries susceptibility	high	undetermined	undetermined	undetermined
Number of rats	51	44	84	54
Number of litters	11	10	12	8
Ave. number of rats per litter	4.6	4.4	7.0	6.7
Rats with caries	41	12	18	9
Rats with caries (%)	80.3	27.3	21.4	16.6
Carious teeth per carious rat	5.5	4.1	2.4	1.9
Carious teeth per carious rat (%)	45.8	34.1	20.0	15.8
Ave. number of carious areas per carious rat	12.5	3.9	3.4	1.6
Caries score per carious rat	33.6	13.1	5.4	4.1

[1] National Institutes of Health.

[2] Diet 560: Cerelose 72.6%, vitamin-free casein 20.2%, Wesson oil 3.0%, Osborne and Mendel salt 4.0%, thiamine 2.0 p.p.m., riboflavin 3.0 p.p.m., pyridoxine 2.5 p.p.m., calcium pantothenate 20.0 p.p.m., choline 1000.0 p.p.m., inositol 250.0 p.p.m., weekly supplement of vitamin A and D concentrate.

560 started at pregnancy vs. its effect starting at birth. The animals exhibiting dental caries were not more numerous, nor was the severity of caries greater, in experiment 6 than in experiment 5. So far as these results indicate, therefore, this high sugar diet, even though given during pregnancy, did not increase the incidence of caries. Carious lesions in these rats were approximately the same in number and intensity as when the diet was started at the time the young were born.

DISCUSSION

In comparison with our previous studies (McClure, '45), the production of caries in white rats was much more satisfactory if a diet containing a large amount of cerelose was given to young rats immediately following birth rather than after weaning. This result is in agreement with the work of Mitchell and Shafer ('49) who, as previously mentioned, found that caries in hamsters was most marked when the diet was started 8 to 30 days after birth. Our result agrees also with Sognnaes' finding ('47b) that more caries developed if a sucrose diet was started during lactation instead of posteruptively.

The most striking effect of starting the cariogenic diet at birth occurred with diet 561 (experiment 1). Obviously this diet is less highly purified than diet 560, owing to the weekly addition of raw carrots to diet 561. Protein and minerals are probably adequate but some vitamin supplies may be inadequate. It is impossible at this time, however, to attribute any particularly cariogenic property to this diet or to evaluate any effect of this diet specifically on the development of young rats during lactation.

The results with the control diet 560 started at birth (experiments 3, 4 and 5) were most striking in experiment 3, involving the group of highly caries susceptible rats. However, the results in experiments 4 and 5 were a distinct improvement over previous results (McClure, '45) in which excessive sugar diets were started at weaning age.

It is believed that much may be said for starting a cariogenic diet at birth. Young rats may start eating solid food

at 15 to 18 days of age and thereby expose their teeth to an excessive amount of sugar during and immediately following tooth eruption. All the rats were sacrificed at 120 days of age, but the fact that rats which started eating at 15 to 18 days were on the diet a few days longer than the rats started at weaning would not account for the caries differences.

Although the data generally apply to rats beyond weaning age, it has been shown previously that age has an influence on the development of caries in the rat (Braunschneider et al., '48), the teeth of rats being significantly more resistant to decay at 100 days and 150 days than at 35 days. Hodge ('43) has also reported that weaning rats developed caries more rapidly than rats which were one year old. In this connection also there is evidence of a decrease in caries susceptibility with age in human teeth (Burrill, '43). A similar relation may be surmised for rats' teeth. In the light of the evidence in this experiment and in the other studies already cited, it would appear reasonable to believe that young and newly erupted molar teeth may be particularly susceptible to a caries-inducing agency, such as a diet containing an excessive amount of sugar.

A role of refined sugars in rat caries etiology is again indicated by our studies. This is in agreement with previous results by other investigators using rats (Sognnaes, '49; Schlack et al., '49), particularly the cotton rat (Shaw, '50; Shaw et al., '44; Schweigert et al., '45), and hamsters (Keyes, '46). It may be suggested at this point, however, that although the results with refined sugar diets have been highly significant, it is to be regretted that the number of animals per group in many of these experiments has been quite small, and also that the published data in many instances give no indication of the incidence of caries per group. The entire caries effect of a diet cannot be evaluated without some knowledge as to the number of rats which develop caries out of a large litter-balanced group. The fact that the prevalence of caries in the human population in this country is about 95% is as impressive in terms of the dental caries problem as are data relative to the number of decayed, missing and filled teeth per person.

The extensive caries which resulted in the caries-susceptible rats in experiment 3 (table 2) emphasizes again the importance of inherent susceptibility in animal caries studies. It is to be noted with regard to experiment 3 that whereas Hunt and his co-workers ('50) used a ground rice diet in their inheritance studies, our results show that caries in their susceptible rats may be produced also by feeding a finely powdered synthetic diet (diet 560).

SUMMARY

Diets containing 72% and 75% cerelose produced a significant incidence of caries and relatively severe caries in white rats, particularly in rats of known caries susceptibility and when the diet was started at birth rather than at weaning. An explanation of the effect of the age at which the dietary change was initiated may be that newly erupted rats' teeth are particularly susceptible to caries attack by a diet high in sugar. The results support the view that for the control of dental caries in children it may be particularly advantageous to restrict the sugar intake.

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THE FOLIC ACID AND VITAMIN B_{12} CONTENT OF THE MILK OF VARIOUS SPECIES[1]

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INTRODUCTION

Although the vitamin content of milk has been studied extensively, very little information is available regarding the vitamin B_{12} content of milk. Cary ('42) reported the occurrence of an unknown factor, X, in cow's milk, and recently Hartman, Dryden and Cary ('49) demonstrated that crystalline vitamin B_{12} possesses complete factor X activity. Differences in the vitamin B_{12} content of milk from different species had not been reported until Collins, Dietrich and Elvehjem ('50) showed that the lower nutritive value of goat's milk could be improved to equal that of cow's milk when vitamin B_{12} and folic acid were added to the former. Preliminary analyses indicated a much lower vitamin B_{12} level in goat's milk than in cow's milk. An extensive study by Hodson ('49) has confirmed earlier observations that cow's milk is a poor source of folic acid, but similar information is not available for the milk of other species.

This investigation was initiated in order to extend the limited knowledge of the comparative folic acid and vitamin B_{12} content of milk from various species.

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EXPERIMENTAL

The milk samples used in this study were obtained from the University Farms and the Wisconsin General Hospital. Brief descriptions of the samples are included in the footnotes to the tables presented.

The milk samples from the cows and goats were taken as aliquots from the mixed milk of a single milking; the samples from all other species were obtained while the young were being nursed. All samples were collected in 100-ml brown bottles and refrigerated immediately at 4°C. after the addition of toluene. Most of the samples were assayed within two days for both folic acid and vitamin B_{12}, using microbiological methods.

Vitamin B_{12} activity was measured using *Lactobacillus leichmannii* American Type Culture Collection 4797 and the medium of Thompson, Dietrich and Elvehjem ('50). Folic acid was determined using *Streptococcus faecalis* R and the medium of Henderson and Snell ('48), modified by replacing the amino acid mixture with acid hydrolyzed casein (1 gm/100 ml of double strength medium). The turbidity of the milk made the use of the turbidimetric method infeasible; hence the acidimetric method was adopted, using a total volume of 2 ml.

The occurrence in milk of vitamin B_{12} which was unavailable to the microorganism was considered, but since little or no increase in the vitamin B_{12} activity of either cow's milk or goat's milk was observed after incubation with trypsin (Thompson et al., '50), all determinations were made directly on diluted whole milk samples. The values observed using this method agreed well with the values for cow's milk and goat's milk obtained by the vitamin B_{12} rat assay of Register et al. ('49).

Since the extensive study of Hodson ('49) demonstrated that no large amount of the conjugated forms of folic acid occur in cow's milk, only free folic acid was determined.

At the beginning of this study a number of samples had to be stored for some time, and since the folic acid values on

these samples were erratic, bacterial synthesis was suspected. Hodson ('49) reported that incubation of cow's milk samples at 37° or 45°C. for 12 to 18 hours produced large increases in folic acid. In order to determine the extent of possible changes in folic acid and vitamin B_{12} activity during storage, 7 samples of goat's milk, with added toluene, were held at 4°C. for 4 weeks and were assayed weekly for these vitamins.

The values shown in table 1 indicate that both the folic acid and vitamin B_{12} activities of the stored samples increased. The folic acid level increased greatly after one week, whereas the vitamin B_{12} level did not increase noticeably until two

TABLE 1

Variations in the folic acid and vitamin B_{12} content of goat's milk samples which were stored at 4°C. after the addition of toluene

DAYS OF STORAGE	FOLIC ACID		VITAMIN B_{12}	
	Mean	Range	Mean	Range
	μg/l	μg/l	μg/l	μg/l
0	1.2	0.6– 2.0	0.11	0.07–0.15
7	8.6	2.7–17.0	0.08	0.05–0.15
14	8.9	5.0–15.0	0.25	0.1 –0.5
21	9.4	1.9–32.0	0.21	0.12–0.4
28	8.8	2.6–24.0	0.27	0.22–0.4

weeks of storage. The pH of the samples dropped markedly, indicating the presence of acid-producing microorganisms. The wide ranges and extreme variability observed demonstrate that storage at 4°C., even with added toluene, does not prevent synthesis of folic acid and vitamin B_{12} by the milk flora. Autoclaving or freezing milk samples to prevent bacterial action caused precipitation of protein; therefore it was necessary to assay the milk shortly after collection in order to obtain homogeneous samples with unaltered vitamin content.

The semi-micro Kjeldahl method was used for the determination of total nitrogen.

RESULTS AND DISCUSSION

The mean values and ranges for the folic acid, vitamin B_{12} and nitrogen content of milk from 10 Saanen goats during the course of lactation are presented in table 2. Similar values for a single Guernsey cow are included for comparison. The ranges give an indication of the individual variations observed in these animals. In every instance the colostrum from both the goats and cow was higher in vitamin B_{12} and folic acid than the regular milk. The folic acid content of milk from both species occurs at a comparable level and falls to a relatively constant value several days following parturition. The vitamin B_{12} content of cow's milk is from two to 50 times higher than that of goat's milk, depending upon the stage of lactation. This confirms the work of Collins et al. ('50) regarding the relative nutritive values of the two milks in rat feeding experiments.

A study of the vitamin B_{12} content of milk from Holstein, Guernsey and Jersey cows (table 3) demonstrated no noticeable breed differences; however wide individual variations were observed. The cows in this study received cobalt as a component of the trace-mineralized salt mixture in their ration. Since it has been observed (Abelson and Darby, '49) that cobalt stimulates the synthesis of vitamin B_{12} by the rumen flora, it is conceivable that the vitamin B_{12} content of milk may be higher in herds receiving trace-mineralized salt.

The vitamin B_{12} and folic acid content of human milk are presented in table 4. Due to the present interest in the roles of vitamin B_{12} and folic acid in human nutrition, individual values for human milk samples are presented. The large variation observed in the vitamin B_{12} content of this milk is probably the result of variations in the dietary intake of the vitamin. The rather low vitamin B_{12} level may suggest a relatively low requirement of vitamin B_{12} for the normal infant.

A summary of the folic acid and vitamin B_{12} content of the milk of all animals studied is presented in table 5. Although some 400 milk samples from 8 different species were assayed

TABLE 2

The vitamin B_{12}, folic acid and nitrogen content of milk from the Saanen goat and the Guernsey cow

POST-PARTUM	SAANEN GOAT [1]					GUERNSEY COW [2]		
	Vitamin B_{12}		Folic acid [3]		Nitrogen	Vitamin B_{12}	Folic acid	Nitrogen
	Mean	Range	Mean	Range	Mean			
	µg/l	*µg/l*	*µg/l*	*µg/l*	*gm/100 ml*	*µg/l*	*µg/l*	*gm/100 ml*
0 days	4.2	1.3 –8.5 (5)[4]	18	11 –30 (5)[4]	1.48 (5)[4]	16	7.5	1.24
1 day	1.2	0.40–2.2 (9)	8.2	4 –15 (9)	0.76 (9)	15	2.0	0.75
2 days	0.55	0.30–1.1 (8)	5.4	2 –13 (9)	0.63 (9)	14	1.1	0.74
3 days	0.37	0.25–0.60 (8)	4.0	1.5 – 6.0 (8)	0.63 (8)	..	..	...
4 days	0.42	0.27–0.90 (8)	3.3	1.2 – 6.0 (7)	0.61 (8)	15	1.0	0.68
5 days	0.41	0.25–0.75 (9)	3.6	0.60– 7.8 (9)	0.59 (9)	13	1.2	...
6 days	0.41	0.25–0.50 (8)	4.5	1.1 – 8.2 (7)	0.56 (8)	11	1.0	0.63
2 weeks	0.29	0.13–0.50 (9)	3.0	0.60– 9.0 (8)	0.52 (9)	7.5	1.1	0.55
3 weeks	0.18	0.08–0.40 (8)	3.2	1.1 – 7.0 (7)	0.49 (9)	7.3	1.6	...
4 weeks	0.14	0.10–0.22 (6)	2.7	1.2 – 5.0 (7)	0.46 (8)	8.8	1.0	...
6 weeks	0.16	0.08–0.30 (7)	1.8	0.60– 3.5 (7)	0.44 (6)	8.4	4.5	...
8 weeks	0.13	0.09–0.18 (7)	2.3	0.80– 4.2 (7)	0.46 (3)	7.1	3.5	...
10 weeks	0.11	0.08–0.14 (8)	4.2	1.2 – 5.7 (4)	...	7.5	1.8	...

[1] The goats were kept in a dry lot and fed a ration consisting of a grain mixture, alfalfa hay and corn silage with no added trace minerals.

[2] The grain mixture fed this cow contained 1% of trace-mineralized salt.

[3] The goat's milk samples for the first three weeks were stored longer than one week before being assayed for folic acid; therefore the higher folic acid values for the ranges during this period probably reflect some bacterial synthesis.

[4] Figures in parentheses represent the number of animals.

for folic acid, all the mean values were within the range of from 1 to 4 μg per liter. The folic acid level of human milk was consistently lower than that of the other milks studied.

In the milk of the ruminants, the cow, sheep and goat, individual variations of vitamin B_{12} were quite large, yet definite species differences were observed. Dietary cobalt has been mentioned as a factor which may possibly influence the vitamin B_{12} content of the milk of ruminants; however, neither the sheep nor the goats used in this study received trace minerals, therefore the difference observed between these two

TABLE 3

The folic acid and vitamin B_{12} content of milk from the Holstein, Guernsey and Jersey breeds of cattle

(The samples were obtained at weekly intervals from 4 cows of each breed)[1]

VITAMIN	WEEK	HOLSTEIN		GUERNSEY		JERSEY	
		Mean	Range	Mean	Range	Mean	Range
		μg/l	μg/l	μg/l	μg/l	μg/l	μg/l
Vitamin B_{12}	1st	7.4	4.9 – 9.2	8.7	7.0 –11.0	8.7	7.0 –11.0
	2nd	9.2	7.0 –12.0	5.8	4.4 – 6.8	6.3	3.2 – 9.0
	3rd	3.8	3.2 – 4.6	5.4	4.2 – 7.4	4.5	4.1 – 5.0
Folic acid	1st	1.5	1.0 – 2.7	2.1	1.3 – 3.5	1.0	0.60– 1.4
	2nd	0.60	0.55– 0.70	1.1	0.70– 1.8	0.50	0.20– 1.0
	3rd	1.6	0.60– 4.0	1.8	1.0 – 2.8	1.2	0.80– 1.8

[1] The cows were on pasture and were fed a grain mixture containing 1% of trace-mineralized salt.

species is not due to this factor. Unfortunately all the cow's milk samples analyzed were from animals receiving trace minerals.

The vitamin B_{12} content of the milk of the non-ruminants studied was highly variable. One important factor affecting the level of this vitamin in these milks appears to be the dietary intake of vitamin B_{12}. The lower values presented in the ranges for the sow, rat and dog represent milk from animals maintained on all-plant rations which were believed to be low in vitamin B_{12}, while the higher values of these ranges represent milk from animals receiving supplements contain-

TABLE 4

The vitamin B_{12} and folic acid content of the milk from 5 human subjects (micrograms per liter)

DAYS POST-PARTUM	1		2		3		4		5	
	B_{12}	Folic	B_{12}	Folic	B_{12}	Folic	B_{12}	Folic	B_{12}	Folic
3	0.45	0.30			0.10	[1]	0.30	[1]		
4	0.35	0.10	0.85	1.5	0.10	0.25	0.40	0.50	0.24	0.25
5	0.40	0.30	1.5	0.50	0.10	[1]	0.62			
6	0.50	0.15								
7	0.50	0.25			0.05					
8					0.03		0.70			
19									[1]	1.5
20									[1]	0.90
21									[1]	1.8

[1] No measurable quantity.

TABLE 5

The vitamin B_{12} and folic acid content of the milk of various species

		DAYS POST-PARTUM	VITAMIN B_{12}		FOLIC ACID	
			Mean	Range	Mean	Range
			$\mu g/l$	$\mu g/l$	$\mu g/l$	$\mu g/l$
Ruminants [2]	Cow [1]	90–150	6.6	3.2 –12.4 (12)	1.3	0.20–4.0 (12)
	Sheep [3]	80–100	1.4	1.0 – 2.0 (9)	2.2	0.80–5.6 (9)
	Goat [3]	30–60	0.12	0.07 –0.18 (10)	2.7	0.60–5.7 (10)
Non-ruminants [2]	Human	3–21	0.41	0.10 – 1.5 (10)	0.71	0.10–1.8 (10)
	Sow	1–30	1.05	0.03 [5]– 2.7 (19)	3.9	1.2 –7.5 (4)
	Rat	3–21	...	11.0 [5]–95 [6] (5)	..	2.0 –3.5 (2)
	Dog	3–6	...	0.70 [5]–13.0 (2)	..	1.5 –3.7 (2)
	Horse	10	0.02	(1)	1.3	(1)
Commercial cow's milk [4]	Raw		3.4	2.4 – 4.6 (5)		
	Pasteurized		4.0	1.6 – 6.5 (12)	2.6	1.5 –5.0 (6)

[1] Trace-mineralized salt fed.

[2] The numbers in parentheses indicate the number of animals studied.

[3] No trace-mineralized salt fed.

[4] The numbers in parentheses indicate the number of samples studied.

[5] The lower values in these ranges were milk samples from animals fed all-plant rations.

[6] An addition of 10 μg of vitamin B_{12} per 100 gm of ration was made to the all-plant diet.

ing vitamin B_{12}. This observation may also explain the low value obtained for mare's milk. Of particular interest is the extremely high level observed in rat's milk.

In view of the effect of dietary vitamin B_{12} upon the level of this vitamin in the milk of non-ruminants, average values probably are meaningful only in rigidly controlled experiments.

The folic acid and vitamin B_{12} content of commercial cow's milk are presented at the bottom of table 5. The vitamin B_{12} level of these milk samples approached the range observed for the herd study presented in table 3. On the basis of these data, commercial milk appears to be a good source of vitamin B_{12}.

SUMMARY

The free folic acid and vitamin B_{12} content of milk from the cow, goat, sheep, rat, dog, sow, horse and human were determined microbiologically. The mean folic acid level of the milk from all 8 species was found to vary from 1 to 4 μg per liter. The colostrum of the cow and the goat was observed to be higher in both folic acid and vitamin B_{12} than the regular milk of these species. The average vitamin B_{12} content of the milk from the cows, sheep and goats studied was 6.6, 1.4 and 0.12 μg per liter, respectively. No breed differences were observed in the vitamin B_{12} content of the milk from the Holstein, Guernsey, or Jersey cows. Commercial cow's milk was found to contain 1.6 to 6.5 μg of vitamin B_{12} per liter.

The vitamin B_{12} content of the milk of the non-ruminants was observed to be more variable than that of the ruminants. These variations were in part due to the dietary intake of this vitamin. The mean folic acid and vitamin B_{12} content of the milk from 5 human subjects were found to be 0.71 and 0.41 μg per liter.

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SEQUELAE TO THE ADMINISTRATION OF VITAMIN B_{12} TO HUMANS [1]

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THREE FIGURES

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This communication will report some observations on the metabolism and fate of orally and parenterally administered vitamin B_{12}, and on its effect on nitrogen balance in children and on the weight gain of growing children during supplementation of an adequate diet with the vitamin.

GENERAL VITAMIN B_{12} EXPERIMENTS

Metabolism and fate of vitamin B_{12}

Chow and associates (Chow et al., '50) have shown that vitamin B_{12} activity could not be found in the urine of normal individuals or untreated pernicious anemia patients following oral administration of a large dose of 500 μg. Subsequently this lack of B_{12} activity in urine was seen in several individuals receiving as much as 5.0 mg of the vitamin orally (Conley et al., '51). In both types of experimental subjects, striking increase in B_{12} activity in the urine followed a single intramuscular or intravenous injection of 50 to 1000 μg, and the recovery of the injected vitamin was essentially complete. In growing children (see below), we have noted that even

[1] The author wishes to express appreciation to Dr. Francis Schwentker and Dr. B. S. Joslin for their assistance in the several portions of this report. As additional observations are accumulated, these sections will be reported *in extenso* in a series of joint publications.

after 6 months on B_{12}-supplemented diets (daily oral dose, 25 μg) virtually no detectable B_{12} activity was found in the urine. To follow further the fate of vitamin B_{12} after oral administration, we have measured B_{12} activity in the feces of a number of normal individuals before and after administration of a test dose. The results of a typical experiment are given below.

Four males, clinically healthy by the usual criteria, were studied. Preliminary fecal collections were made during two 24-hour periods on each of the subjects. Immediately thereafter, each subject received orally a test dose of 2 mg of crystalline vitamin B_{12}, and feces were collected for another two 24-hour periods. Each fecal sample was homogenized in a Waring Blendor within two hours after defecation and diluted with water to 3 l. Aliquots were autoclaved at 15 pounds pressure for 15 minutes. Upon cooling, they were centrifuged and supernatant fluids were used for assay. Microbiological estimation of B_{12} activity was made on portions of the supernatant liquids, with *L. leichmannii* as the test organism (Skeggs et al., '50). All measurements of B_{12} activity are reported as vitamin B_{12}; it may be likely, but it is not necessarily true, that such assay techniques specifically measure vitamin B_{12}. In preliminary experiments it was found that reproducible results could not be obtained without autoclaving. The data, given in table 1, show a marked increase in vitamin B_{12} in the feces 24 hours following oral administration of doses of 2.0 mg.

These results suggest that more than half of the orally administered B_{12} is either excreted unchanged or modified during passage through the gastrointestinal tract in a way which does not obliterate microbiological activity. These data are in agreement with the findings of Chow and associates ('51), who fed orally a massive dose of a vitamin B_{12} preparation (Chaiet et al., '50) containing radioactive Co^{60} to a group of rats and recovered over 50% of both the microbiological activity and radioactivity in the feces but only a small fraction of 1% in the urine.

Since it seems likely that a requirement for vitamin B_{12} exists in humans and is normally satisfied by the vitamin B_{12} content of foodstuffs, certain alternatives are open for consideration. B_{12} activity in foodstuffs may reside in unknown complexes of the vitamin which are absorbable by most people. There may be a limit to the extent to which material responsible for B_{12} activity, if it is not pure B_{12} itself, is absorbed, and this limit may be close to the level at which it is metabolized to yield inactive urinary excretion products; the balance of an oral dose passes through the gastrointes-

TABLE 1

The appearance of an increase in vitamin B_{12} activity in the stool of subjects fed the vitamin by the oral route

HOURS FROM ADMINISTRATION	MICROGRAMS [1] B_{12} FOUND	EXCRETION [2]
		%
—48	30	0
—24	30	0
24	1130	55
48	1330	65

[1] Micrograms of vitamin B_{12} found per 24 hours (average of 4 subjects). No marker was given.

[2] Cumulative per cent of the ingested vitamin excreted (average of 4 subjects; total excretion minus pre-administration excretion).

tinal tract either unchanged or metabolized or modified by the bacterial flora to yield only a fraction of the ingested activity in the feces.

The distribution of vitamin B_{12} in blood

Earlier results (Conley et al., '51) indicated that about 8 hours after intramuscular administration of a solution containing 1.0 mg of vitamin B_{12} to normal human subjects, blood levels of vitamin B_{12} were essentially nil; most of the injected B_{12} was found in the urine. Samples of citrated blood taken about 15 minutes after injection, however, contained

as much as 29 to 34 mμg of vitamin B_{12} per milliliter of blood, while the corresponding plasma samples contained 50 to 80 mμg. These results suggest that vitamin B_{12} does not penetrate into or is not adsorbed onto the red cells within 15 minutes after intravenous administration.

To ascertain whether vitamin B_{12} could penetrate the erythrocytes on prolonged incubation, we added 50 μg of vitamin B_{12} in 0.10 ml of 0.95% NaCl solution to 10.0 ml of freshly defibrinated human blood. The suspension was incubated at 37°C. for three hours under essentially sterile conditions and then centrifuged in an angle centrifuge for 30 minutes. Plasma was carefully removed with a fine capillary. The packed cells (about 5.0 ml) were washed with

TABLE 2

The distribution of vitamin B_{12} in blood (in vitro)

FRACTIONS	B_{12} FOUND	RECOVERY OF B_{12}
	μg	%
Laked erythrocytes	0.05	0.1
Washings (combined)	4.8	9.6
Plasma	62.5	125.0

1.5 ml of an isotonic saline solution and recentrifuged. Washing with the same amount of liquid was repeated twice more. The cells were then laked with water and diluted to 20 ml. The plasma sample, the combined washings and the laked blood cells were assayed separately for vitamin B_{12}.

The resulting data are given in table 2. Virtually all of the added vitamin was in the plasma, but a small fraction, as would be expected, was found in the washings. Approximately one-tenth of 1% of the vitamin was accounted for in the laked cells. If the vitamin B_{12} were able to penetrate into the erythrocytes under our experimental conditions, the concentration of this vitamin in the plasma should be approximately one-half of that actually found by analysis, since the hematocrit reading was 45% and that in the laked cells

should be many-fold higher. These data demonstrate that vitamin B_{12} does not diffuse into the red cells even upon incubation for three hours at 37°C. There was no evidence of the presence of any enzyme in blood capable of destroying vitamin B_{12}, since the total activity found in the three fractions slightly exceeded the amount of B_{12} added, this difference lying within the experimental error of our routine assay.

Effect of vitamin B_{12} on nitrogen balance in infants

It is generally agreed that vitamin B_{12} is closely associated with the so-called "animal protein factor." Supplementation of a diet containing only vegetable proteins with vitamin B_{12} increased the "feed efficiency" of the diet for pigs (Cunha et al., '50); i.e., less feed was required to produce a given amount of weight gain. This increase in "feed efficiency" does not necessarily indicate an improvement in the utilization of the vegetable proteins in the diet, since these investigators did not measure the retention of nitrogen in the diets with or without supplementation. Chow and Barrows ('50) have shown that in B_{12}-deficient rats the addition of vitamin B_{12} to diets whose sole source of protein was obtained from soybeans failed to enhance the retention of nitrogen; i.e., the per cent of ingested nitrogen retained. These findings suggest, in the light of the observed greater growth of the supplemented animals, an effect of the vitamin on either carbohydrate or fat metabolism, rather than protein metabolism. It has been shown that there is an interrelationship between vitamin B_{12} and dietary fat in mice (Bosshardt et al., '50) and in rats (McCollum and Chow, '50). These observations are, therefore, complementary, and tend to rule out an effect of B_{12} on protein metabolism. It becomes important to extend these findings to humans to ascertain whether a species difference exists and whether vitamin B_{12} supplementation will enhance nitrogen retention in infants fed a diet deficient in this vitamin.

For this phase of our study 4 infants (three males and one female, three to 6 months of age) were offered ad libitum a

basal diet consisting of 100 gm of a soybean preparation and 40 gm of Dextri-maltose No. 1 in water to a final volume of 750 ml. A supplement [2] of a multivitamin emulsion was administered daily. It was thought that feeding these infants soybean meal as the sole source of dietary proteins might deplete them of any reserve of vitamin B_{12}, and therefore they would be more likely to respond to subsequent administration

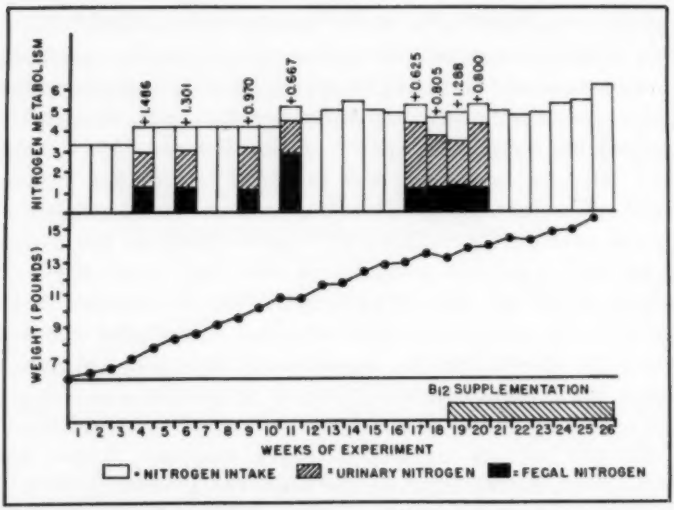

Fig. 1 Nitrogen balance studies on a male infant (GN), 6 weeks old at the start of observation.

of this vitamin. Throughout this time the infants were weighed regularly and nitrogen balance studies were carried out for several weekly periods. At the end of the preliminary period of three months daily supplementation with vitamin B_{12} (10 μg, intramuscularly) was begun, and the nitrogen balance studies continued. Since the values obtained with all subjects were essentially the same, data for one, typical of the group, are shown in figure 1.

[2] These supplements were supplied for this study by Mead Johnson and Hoffmann-LaRoche under the trade names Polyvisol and Vi-Penta, respectively.

The data fail to offer any suggestion that vitamin B_{12} supplementation increases nitrogen retention or the fraction of ingested nitrogen retained. There is no evidence, however, that maintenance of these children on the soybean diet had actually stripped out vitamin B_{12} reserves, or that any deficiency existed prior to supplementation. If the vitamin has any effect on protein utilization, such a role is not shown under the conditions of these experiments. It seems safe to conclude that vitamin B_{12} supplementation, at the levels administered, did not modify protein utilization, as measured by nitrogen balance data, in these subjects.

Growth response of growing children to B_{12} supplementation

It is well-established that the growth rate of a variety of animals, such as rats (Emerson et al., '49), mice (Bosshardt et al., '50), hogs (Neumann et al., '50), and chicks (Ott et al., '48) on a diet deficient in vitamin B_{12} is increased if vitamin B_{12} is added to the diet. Accumulated experience suggests that the enhanced growth rates are reflections of increased food intake ascribable to appetite stimulation.

Whether the vitamin accelerates the growth of children is difficult to decide on the basis of the published reports of Wetzel and his associates ('49) and of Downing ('50). The first group of investigators administered a daily oral dose of 10 μg of vitamin B_{12} to a group of 11 children from a fresh air camp for a period of several weeks. Other resident children, to whom no supplement was given, served as controls. The results led these workers to conclude that this vitamin is effective in promoting the growth response of children with retarded growth.

In the second study (Downing, '50), vitamin B_{12} was given intramuscularly to a group of 25 premature infants. Twenty-three others served as controls. No differences in average total gain before discharge, average daily gain or average time required to reach a weight of 2.5 kg were observed. In both of these investigations, as in the one reported here, it

was not known whether there was any pre-existing deficiency of the vitamin, or lack of it in the diet during the experimental period.

We have been interested in ascertaining whether vitamin B_{12} supplementation can stimulate the weight gain of children receiving a normal diet ad libitum, with substantial supplements of other known vitamins.

It seemed likely, *a priori,* that more striking effects, if they were to be obtained, would be seen in subjects presenting some history of growth retardation as a result of malnutrition or chronic illness. For this reason major emphasis has been placed on accumulation of data from such children but, to round out the picture, some observations have been made on clinically normal children as well. These preliminary observations are reported in this communication in order to bring out the problems peculiar to this type of study and to show the trend of, but not to draw definite conclusions from, our observations on the effects of vitamin B_{12}.

EXPERIMENTAL PROCEDURE FOR THE STUDY WITH CHRONICALLY ILL CHILDREN

Experimental subjects

All the subjects studied in this phase of the program were in a convalescent home. They suffered from chronic illness, such as rheumatic heart disease, simple malnutrition or anemia, minor physical defects, or mental retardation.

Design of study

Before the start of the study, detailed instructions were given to the head nurse in charge with regard to the distribution and care of the prospective subjects. According to the plan, all children, on admission, were numbered consecutively. It was decided in advance that those with even numbers were later to receive vitamin B_{12} supplementation; those with odd numbers would constitute the control group. This method of alternation decided at the time of admission in

which group each child would later fall, without regard to sex, age or disease. Each child was observed for 6 weeks, during which time adjustment to new routines and environment occurred. A majority of these children gained a significant amount of weight during the first two weeks of this period. In most cases the children's weights remained essentially constant during the 4 following weeks. Therefore, 6 weeks after admission the period of experimental observation was begun.

All children were at that time carefully weighed. These weights were considered as the base weights with which the subsequent records were to be compared. All of the subjects received the regular home diet throughout. The children in the control group received no supplement, while those in the experimental group were given an oral supplement of vitamin B_{12}. No attempt was made to give the control group any placebo, since all children in both groups were receiving a variety of medicaments in tablet form as part of their specific treatments. One tablet [3] containing 25 μg of vitamin B_{12} was given daily to children (6 years or older) who were able to swallow it. The younger children in the experimental group, who could not be depended upon to swallow tablets, received daily an equivalent amount of this vitamin in solution.[4] For infants below two years of age, a solution providing 10 μg of crystalline vitamin B_{12} [5] was administered each day by dropper. All children were weighed at regular intervals. The weights of the infants (three years or younger) were recorded every other day to the nearest ounce; children in the older group were weighed weekly to the nearest 4 ounces. The identical scale was used for any one individual during the study. At the time of weighing previous records were not available to the weighers, nor did they know whether any particular subject was receiving the supplement.

[3] Rubramin tablets and Rubramin solutions were supplied by E. R. Squibb and Sons.

[4] See footnote 3.

[5] Crystalline vitamin B_{12} (Cobione) was supplied by Merck and Company.

Dietary regime

All subjects were given food ad libitum. For the infants, care was exercised not to limit their daily milk formula intake. Children in the older group were allowed to have as many helpings of food as they desired, although no persuasion was ever employed. Menus were uniformly well-balanced and diversified as to content. Food consumption data were not accumulated and, as a consequence, it is not possible to estimate reliably the daily caloric intakes or the distribution of calories among protein, carbohydrate and fat. The formula for infants provided milk, Dextri-maltose and water. The vitamin supplements[6] for all children were those regularly used in the home and provided ample amounts of vitamins A, D, B_1, C, and niacin amide but no known source of vitamin B_{12}.

Results

During a study which lasted about one year, a total of 96 subjects were admitted to this home. Out of this number, 24 subjects in the control group and 21 subjects in the vitamin B_{12} group stayed three months or longer following the initial adjustment period of 6 weeks. Records of the weight changes of the others are not included in this report because of insufficient duration of observation. For the 45 children comprising the experimental population, distribution by diseases and by sex and age are given in tables 3 and 4, respectively. In the distribution according to diseases, we find that there were almost equal numbers of children suffering from malnutrition in both groups, but the number of children in the control group with mental retardation or minor physical defects was greater than that in the B_{12} group. Probably a more important difference in the distribution of diseases between the two groups lies in the fact that the B_{12} group contained more children with rheumatic fever or congenital heart disease than the control group. Whether these differences will

[6] See footnote 2, page 328.

eventually change the trends our present data reveal cannot be answered at this time.

From the sex-age distributions (table 4), it would appear that the two groups of males were somewhat balanced against each other. The age distributions of the females are not

TABLE 3

Distribution of diseases

CATEGORY OF INTEREST	RECEIVED B_{12}	CONTROL
Malnutrition	10	11
Mental and physical defects	2	6
Rheumatic heart and congenital heart conditions	8	4
Chronic infection	1	3
Total	21	24

TABLE 4

Distribution by age

AGE IN YEARS	MALE		FEMALE	
	B_{12}	Control	B_{12}	Control
0.5–2	1	2	6	2
2 –4	0	2	0	1
4 –6	1	2	1	3
6 –8	3	3	0	3
8 –10	2	3	3	0
10 –12	2	3	2	0
Total	9	15	12	9

similarly comparable, the experimental group having larger numbers of both very young and older children. The numbers in any corresponding pair of cells are too small to permit valid limited range comparisons to be made. In evaluating the over-all data, the age-sex effects have been considered only to a limited extent.

Grossly, the two groups are comparable with respect to mean weights and their ranges; these values are 42.2 pounds (10 to 83.5) for the control group and 43.0 pounds (11 to 78.5) for the B_{12} group. The mean ages and their standard deviations are roughly comparable, being 69.7 $\pm$ 38.8 months, and 79.1 $\pm$ 44.2 months for the control and B_{12} groups, respectively.

The weight gains of all children in each group, after the initial adjustment period of 6 weeks, are given in figure 2.

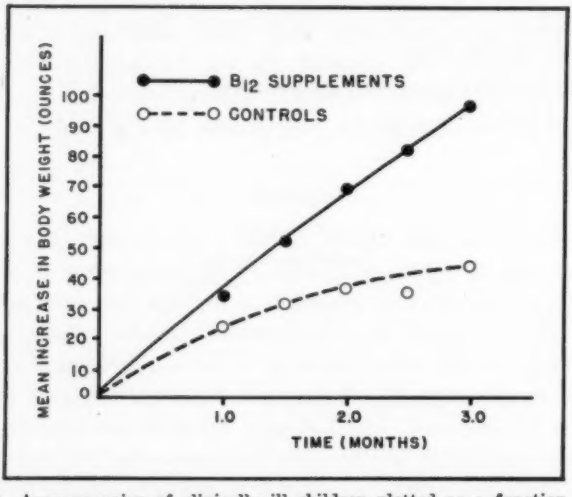

Fig. 2 Average gains of clinically ill children plotted as a function of time, without segregation of the supplemented and control groups with respect to age, sex, or clinical syndrome distribution.

The data demonstrate after three months of vitamin B_{12} therapy the mean gain in body weight in the experimental group was practically twice that in the control group. The difference in mean weight gains began to manifest itself after one month, and consistently increased with time.

At the end of the three months' observation period the difference between the means (53.0 oz.) was statistically very highly significant ($p < 0.001$). The possibility that this re-

sult is a reflection of differences in the age distribution in the two groups is at least partially discounted by the observation that analysis of covariance, using age as the independent variable, does not alter the significance of the difference in the mean weight gains of the two groups, sex difference being ignored.

Moreover, analysis of variance of the accumulated observations at approximately half-month intervals from one month through three months shows an over-all highly statistically significant difference ($p < 0.01$) between the groups, and a highly significant ($p < 0.01$) difference in linear trend with time. Similar analyses of these data following subdivision into two groups according to sex show significant differences ($p < 0.05$) resulting from supplementation and highly significant differences ($p < 0.001$) in linear trend between the supplemented and unsupplemented groups for both males and females. These trend differences support the impression given by figure 2 that the weight changes in the two groups followed markedly diverging trends, which increased with time the separation of weight gains at any given time. Such analyses provide unbiased assessment of the statistical significance of the gross differences, ignoring age, by a method which effectively integrates all of the observations over the period considered. Interpretation from such analyses may be regarded as substantially more meaningful than conclusions drawn from the data at any one point in the course of the study.

Since the subjects here studied represented a variety of clinical conditions, it seems wise to attempt separation by clinical class. Substantial numbers in each of the groups shown in table 3 can be described as malnutrition cases. When data from these individuals are analyzed separately, we have 10 children who received B_{12} and 11 controls. For these groups the weight gains at the end of three months were, respectively, 89.3 and 45.1 oz.; the difference of 44.2 oz. is highly significant ($p < 0.01$). Integrated analysis by the variance technics employed before reveals a highly significant

difference in linear trend ($p < 0.001$) but only a moderately significant difference between groups ($p < 0.05$). This latter may be a reflection of the smaller number of individuals in each group, as well as of the fact that these smaller groups were not well balanced with respect to sex and age distribution.

EXPERIMENTAL PROCEDURE FOR THE STUDY WITH CLINICALLY NORMAL CHILDREN

Experimental subjects

The children studied in this part of our work were resident in a foundling house. The resident population was divided into three age groups (0.5 to 17 months; 18 to 47 months; and 48 to 77 months). At this time, insufficient data on children in the first and third groups are available for statistical analyses. Data on the 18- to 47-month-old group of males only will be reported.

At the time when the study was initiated, there were 24 male children in this home within the age range of 18 to 47 months. The name of each child was recorded on an individual index card. These cards were then shuffled and separated into equal sized control and B_{12}-treated groups by a strictly random procedure.

Design of study

One group received a daily supplement of 25 μg of vitamin B_{12} by addition to milk of 0.5 ml of a solution of 25 mg of the crystalline vitamin [7] in 500 ml of water. The solution was freshly prepared each week.

The control group received similarly a placebo in the form of a solution of 45 mg of vinta certified dye [8] in 2,000 ml of water. The color of the dye solution resembles that of the vitamin solution. These two test solutions were measured in

[7] See footnote 5, page 331.

[8] Vinta dye is a mixture of F. D. and C. certified dyes, supplied by the National Aniline and Chemical Company.

amounts approximately 10 to 15% above the weekly requirements of individual nurses, in two different colored bottles — blue for the vitamin B_{12} solution and brown for the placebo. To minimize further any error in the administration of the solutions, the crib of each infant was also marked with blue or brown corresponding to the color of the bottle from which the supplement was to be administered to its occupant. The solutions were delivered to the nurses weekly.

All children were weighed at a definite hour on a certain day of the week, at which time the weigher was supplied with a mimeographed sheet listing the names of subjects to be weighed, arranged in alphabetical order. At the time of weighing previous records were not available to the weighers, nor were they informed as to whether any given subject was receiving the vitamin or placebo. The records were collected from the weighers on the day of weighing and entered on a master sheet. All weighings on any child were made on the same scale throughout. Weights were measured to the nearest quarter pound.

Results

Population shifts permitted study of only 18 out of the original 24 children over a 24-week period. These children fell into two groups of 9 each which did not differ significantly with respect to age or initial weight. Ages and initial weights for the supplemented and control groups, respectively, were 32.7 ± 8.8 months, 30.6 ± 5.3 pounds; and 32.9 ± 7.8 months, 30.1 ± 4.6 pounds.

The average weight gains of the children in both groups are plotted in figure 3. The mean gain in body weight of the children in the B_{12} group was consistently greater than that of the controls from the 4th week onward. At the end of 24 weeks, the supplemented group showed a weight gain of 58 oz.; the control group, 40 oz. The difference of 18 oz. is statistically significant ($p < 0.05$). Analysis of variance reveals a significant difference in linear trend with time ($p < 0.05$) using biweekly data from the 4th through the 24th

week, but no significant difference in over-all performance between the two groups. It is possible that these findings are fortuitous; nevertheless the consistent trend of the data is strongly suggestive. With an adequate diet, there is no reason to expect very dramatic effects from B_{12} supplementation. If real effects should result from such supplementation, they would be of a small order of magnitude and their statistical evaluation would require additional subjects, or observations over longer periods of time, or both.

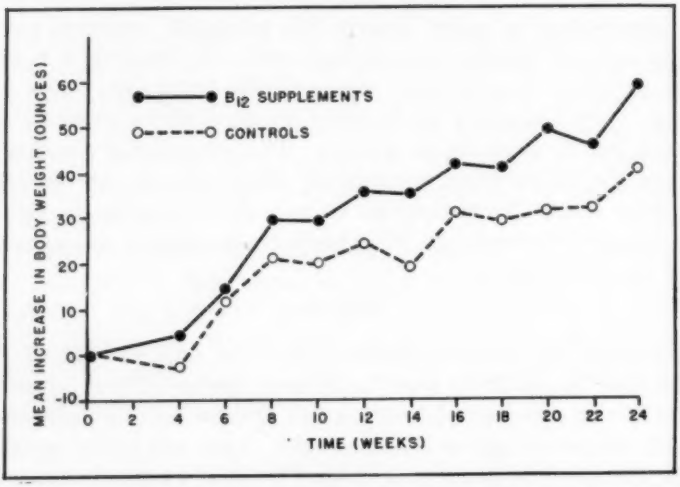

Fig. 3 Average weight gains of supplemented and control groups of clinically healthy male children, 18 to 47 months of age at start of experiment.

Discussion of results

Our data suggest, in general, that vitamin B_{12} may play an important role in the promotion of growth. The greater difference between supplemented and unsupplemented chronically ill children, if it is indeed real and not an artifact, might be conceived as arising from greater deficiency of, or larger requirement for, vitamin B_{12} in such chronically ill children. Our estimates of improved growth rest on greater

weight gains. To the extent that greater weight gain is a criterion of better growth, our findings as to the role of supplementation with vitamin B_{12} in the growth of chronically ill children offer the promise that further study with better balanced groups would clearly point up the benefits to be derived from such supplementation. More data are needed to clarify the situation with respect to normal children.

Our results using chronically ill children are suggestive of a trend of findings similar to that reported by Wetzel and associates ('49). Reconciliation of these findings with those of Downing ('50) is to be sought. It must be recognized, though, that in his study premature infants were used, the period of observation was relatively short, and the published data provide meager bases for evaluating dosage and duration of observations. Furthermore, in the feeding of premature infants it is entirely possible that uncontrollable factors might prevent attainment of the increased food intake necessary to achieve greater weight gains.

The primary objective of the present paper is not to report definitive conclusions on the role of B_{12} as a nutritional factor in the growth of children. We merely wish to report our observations and to point out the problems inherent in the completion of a well-controlled study of this kind. This is timely because of the increasing general interest in the subject, as well as the broader use of oral B_{12} supplements.

In studies such as this many uncontrollable variables inevitably enter the experimental situation. The difficulties encountered in this phase of our study were two-fold. With the chronically ill children the available population was not well balanced with respect to age, sex, or symptomatology, while the data on the well-matched clinically healthy male groups are numerically insufficient. For these reasons the trends apparent in our data are only to be regarded as suggestive; were the experiments less carefully planned it would be impossible to formulate even these tentative ideas.

More important, perhaps, than balancing experimental populations for valid statistical analyses is awareness of the

meagerness of our knowledge of the metabolic role of orally administered vitamin B_{12}. For example, from the published reports on animals and from the data presented in this communication, it is apparent that vitamin B_{12} is not likely to be involved in nitrogen metabolism but may play an important role in carbohydrate or fat metabolism. For this reason, search for the effects of vitamin B_{12} on the growth of any species will result in failure unless the subjects are fed ad libitum. Furthermore, our data demonstrate that the main bulk of orally administered vitamin B_{12} is not absorbed, but excreted in the feces. Questions of dosage and of the route of administration become pertinent. Therefore, the need for studies which might lead to a better understanding of the absorption and utilization of vitamin B_{12} becomes paramount.

In the present investigation we arbitrarily chose a daily oral dose of 25 μg of vitamin B_{12}. There are relatively few data available to serve as guideposts in the choice of dosage in an experiment of this character. In the report by Wetzel et al. ('49), a daily oral dose of 10 μg was employed. Downing ('50) administered the vitamin in smaller doses to premature infants by injection. It has been estimated that the daily oral intake of vitamin B_{12} in what is considered an adequate diet will range from 2 to 5 μg. Our experimental population presented a wide variety of histories from which no conclusions could be drawn as to the possible extent or duration of inadequate dietary vitamin B_{12} intake.

In the treatment of pernicious anemia patients, parenteral administration of 1 μg daily led to desirable clinical response. Oral doses of 5 to 10 μg, together with suitable dietary sources of the intrinsic factor, have also proved satisfactory clinically. In the absence of the intrinsic factor, estimates of the amount of vitamin B_{12} necessary to bring about satisfactory reticulocytosis and clinical improvement have ranged from 25 μg upward to as high as 60 to 100 μg or even 1 mg.

There are no bases for assuming that the dosage necessary for the treatment of pernicious anemia bears any relation to the dosage needed to bring about a growth response of the

type we sought. Some preliminary experiments were carried out with a few children receiving a daily oral dose of 5 μg of the vitamin over a period of two to three months. At this level of supplementation, and on a generally adequate institutional diet, no differences in weight gains were observed during the experimental period. Accordingly, we elected to conduct these experiments using the higher level (25 μg daily of oral vitamin B_{12}), since our purpose was less to establish a dosage level than to seek an effect, even though more critical study, if the effect is established, may subsequently show that this level is excessive.

SUMMARY

1. In confirmation of our previous reports, it was found that oral administration of vitamin B_{12} over a period of several months or in a single massive dose (5.0 mg per adult) did not result in the appearance of vitamin B_{12} activity in the urine. A major portion of the ingested vitamin appeared in the feces. These results indicate poor but apparently adequate absorption of the vitamin in the gastrointestinal tract, even in clinically healthy individuals.

2. When vitamin B_{12} was given intravenously to man, the blood level decreased rapidly and the administered vitamin could be recovered in the urine. Determinations of vitamin B_{12} in plasma and blood failed to yield evidence of penetration into or adsorption onto the erythrocytes within 15 minutes *in vivo*. This was further substantiated in *in vitro* studies, where no appreciable activity could be detected in the red cells obtained from defibrinated human blood previously incubated with vitamin B_{12} for three hours. The vitamin was completely recovered in the plasma.

3. When B_{12} was given parenterally to infants fed on a soybean protein diet, no increase in nitrogen retention was observed.

4. Observations on the effect of vitamin B_{12} on the weight gain of chronically ill and clinically healthy children were made. Data have been presented to show that children of

either type gained greater weight than the corresponding controls if offered B_{12} supplementation in a given period. However, due to the heterogeneity of the subjects with chronic illness and the small numbers of the normal children studied, the results are merely considered suggestive of trends which may be confirmed by extended observations under even better controlled conditions.

ACKNOWLEDGMENTS

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